BACTERIAL METABOLISM OF BENZALDEHYDE

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This is to certify that this thesis is the original work of the candidate done under my supervision and is suitable for submission for the award of Ph. D. degree in Biochemistry.

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Dedicated to

My Parents

A C K N O W L E D G E M E N T

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ABSTRACT

An aerobic organism capable of utilizing benzaldehyde as sole source of carbon has been isolated from the soil by the enrichment culture technique. The organism has been identified to belong to the genus Achromobacter. The cell yield of the organism was maximum at the level of 0.05% (v/v) benzaldehyde. The optimum temperature and pH for growth was 30-35°C and 7.0 respectively.

The pathway for dissimilation of benzaldehyde and related compounds was investigated by the technique of sequential induction. The growth rates and the yield of the organism were measured with benzaldehyde and related compounds.

(i) The benzaldehyde-grown cells oxidized benzaldehyde, benzoate, protocatechuate, catechol, 2,4-dihydroxy benzaldehyde, salicylaldehyde, succinate, malate, fumarate and acetate without lag whereas m-hydroxy benzoate, p-hydroxy benzoate, adipate, citrate and α-ketoglutarate were oxidized with lag. Puromycin decreased the rate as well as total oxygen uptake of

benzaldehyde, p-hydroxy benzoate and m-hydroxy benzoate. Chloramphenicol did not have any effect on the rate of oxidation of benzaldehyde and duration of lag phase in the oxidation of p-hydroxy benzoate and m-hydroxy benzoate. This may be due to non permeability of the antibiotic. o-Nitrobenzoic acid decreased the total oxygen uptake of benzaldehyde as well as the rate of oxidation of p-hydroxy benzoate and m-hydroxy benzoate.

(ii) The benzoate-grown cells oxidised the benzaldehyde, 2,4-dihydroxy benzaldehyde, benzoate, protocatechuate, catechol, succinate and acetate without lag but p-hydroxy benzoate, m-hydroxy benzoate and adipate were oxidised with lag.

(iii) The p-hydroxy benzoate-grown cells oxidized, 2,4-dihydroxy benzaldehyde, p-hydroxy benzoate, protocatechuate, catechol, succinate, acetate and adipate without lag but benzaldehyde, benzoate and m-hydroxy benzoate with lag.

(iv) The m-hydroxy benzoate-grown cells oxidized m-hydroxy benzoate, protocatechuate, catechol, succinate and acetate without lag. Benzaldehyde, benzoate, 2,4-dihydroxy benzaldehyde, p-hydroxy benzoate,

and adipate were oxidized with lag.

(v) The adipate-grown cells oxidized benzaldehyde, protocatechuate, adipate, succinate, acetate and 2,4-dihydroxy benzaldehyde without lag but benzoate, p-hydroxy benzoate and m-hydroxy benzoate were oxidized with lag.

(vi) The acetate-grown cells oxidized benzaldehyde, benzoate, p-hydroxy benzoate, m-hydroxy benzoate, protocatechuate, catechol and adipate with lag. Succinate, acetate and 2,4-dihydroxy benzaldehyde were oxidized without lag.

(vii) The succinate-grown cells oxidized benzaldehyde, benzoate, protocatechuate, catechol, 2,4-dihydroxy benzaldehyde, succinate and acetate without lag but p-hydroxy benzoate, m-hydroxy benzoate and adipate were oxidized with lag.

(viii) Glucose-grown cells oxidized benzaldehyde, catechol, succinate and acetate without lag. Benzoate, p-hydroxy benzoate, m-hydroxy benzoate, protocatechuate and adipate were oxidized with lag. Puromycin and o-nitrobenzoic acid increased the oxygen uptake of benzaldehyde. The rate of oxidation of

p-hydroxy benzoate was effected but the total oxygen uptake was increased in presence of these protein synthesis inhibitors. The increase may be due to the oxidation of inhibitor or some impurity associated with it. The chloramphenicol effected the rate as well as total oxygen uptake of benzaldehyde.

(ix) In the presence of cellular debris, cell-free extracts of benzaldehyde-grown cells oxidized, catechol, protocatechuate, benzaldehyde, benzoate, p-hydroxy benzoate, m-hydroxy benzoate and 2,4-dihydroxy benzaldehyde.

(x) <u>In vitro</u> incubation of benzaldehyde and cell-free extract in presence of $NADP^+$ resulted in the formation of benzoate, p-hydroxy benzoate, protocatechuate. However with protocatechuate or catechol, β-oxoadipate was formed. The p-hydroxy benzoate was also identified in the culture broth.

(xi) The benzaldehyde dehydrogenase was $NADP^+$ dependent and the hydroxylation of p-hydroxy benzoate was NADPH specific.

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III. LIST OF ABBREVIATIONS

NAD^+	...	Nicotinamide adenine dinucleotide (oxidized form)
NADH	...	Nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	...	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	...	Nicotinamide adenine dinucleotide phosphate (reduced form)
FAD	...	Flavin adenine dinucleotide
CoA	...	Coenzyme A

IV. INTRODUCTION

During the past decades, fairly precise knowledge has been obtained of the pathways through which certain microorganisms utilise aromatic compounds. Zobell (1946) has dealt the bacteriological aspects of this phenomenon. The biochemistry of the process has already been reviewed (Happold, 1950; Stanier, 1952). Several types of microorganisms belonging to Coccaceae, Mycobacteriaceae, Bacteriaceae, Pseudomonadaceae, Spirillaceae, and Bacillaceae families are known, which grow aerobically in a simple mineral salt medium with an aromatic compound as sole source of carbon. During the growth of the microorganisms, the benzene ring undergoes fission giving rise to various compounds which are utilized in the diverse processes associated with cell metabolism. Such organisms have been isolated from soil, sewage and mammalian faeces, and are widely distributed in nature. Therefore, biological function of these organisms may be regarded as an essential step in the "Carbon Cycle".

Their use as an industrial scavengers is common, since percolation of phenolic waste products through sewage beds provides a cheap method of detoxicating aromatic compounds potentially harmful to aquatic life. The degradation of aromatic compounds is not only

confined to the bacteria but several fungi like Aspergillus sp. (Kluyver and Van Zijp, 1951); Penicillium sp. (Hockenhell et al., 1952; Isono, 1953; 1954), Oospora sp. (Landa & Eliasek, 1956) and Neurospora sp. (Gross et al., 1956) are capable of such degradation, as well as certain soil (Henderson & Farmer , 1955) and wood-rotting fungi (Fahreus, 1949).

Mostly the carbon skeleton of the aromatic compounds is exclusively converted into carbon dioxide and cell constituents. However, it was first shown that sometimes intermediates are transiently accumulated in the cutlure broth (Evans , 1947). An alternative to this traditional approach is the study of cell-free enzyme preparations made from organisms after growth on the original substrate or any likely intermediate in vitro. From the point of view of origin of the isolated products, the interpretation of results are always difficult. However, the probability of their being real intermediates is vastly increased if labelled compounds are used. This approach has, in almost all cases, always given the initial lead in delineating the intermediary pathways.

The observations, which led to the current

ideas about the factors controlling the enzymatic constitution of a microbial cell, date back to late nineteenth century (Wortmann , 1882). The enzymes produced as a specific response to the presence of the homologous substrate in the culture medium were called " adaptive enzymes " (Karstrom , 1937-38). The constitutive enzymes are always formed by the cells of a given species irrespective of the composition of the medium.

It was observed that the enzymes, of aromatic ring fission elaborated by a particular Pseudomonas sp. were strictly adaptive in nature, because these enzymes were only shown to be present when the organism was grown in presence of an aromatic compound (Stanier, 1947). Stanier, therefore, conceived the idea of using this phenomenon as the basis for a refined type of kinetic analysis to determine the nature of the intermediates that lie on an adaptively controlled metabolic pathway. This valuable technique was called "Simultaneous or Successive adaptation", but for various reasons more accurate terminology of "Sequential induction" has been proposed (Cohn et al., 1953). The principle can be illustrated by considering a hypothetical metabolic pathway, each step of which is catalyzed by a specific inducible enzyme.

$$A \xrightarrow[E_a]{} B \xrightarrow[E_b]{} C \xrightarrow[E_c]{} X \xrightarrow[E_x]{} D \xrightarrow[E_d]{} E \longrightarrow \text{etc}$$

The cells, potentially capable of carrying out above reactions, will be devoid of the relevant enzymes, if they have not been exposed by the conditions of growth to compound A (the inducer). When placed in contact with A, such cells respond by producing the enzyme E_a catalyzing the step $A \longrightarrow B$. The formation of B will in turn provide the necessary activation for the formation of E_b and so on. Thus cells fully induced to dissimilate A will also be conditioned to metabolize B, C, X, D, E etc. On adapting the cells to an intermediate in the chain (say C), they will then also be adapted to the later intermediates, X, D, E etc., but not necessarily to the earlier ones. Consider, however, the case of a compound X, which may appear on chemical grounds to be a possible intermediate in the dissimilation of A, and which is likewise potentially attackable by a specific, inducible enzyme E_x. In view of the known high specificity of the inductive response, it is extremely improbable that cells, specifically adapted to X, shall utilize D, E etc., if X is not a member of reaction chain. Thus, by adapting an organism to a given primary substrate, and then analysing its

adaptive patterns with respect to postulated intermediates, evidence can be obtained as to which of these compounds are actually operative in the reaction sequence. It is, of course, necessary to make a parallel test with "Unadapted" cells grown in the absence of all the compounds under test, in order to make certain that the relevant enzymes are not constitutive. In practice the application of this technique is quite simple. Washed cell suspensions, grown on the substrate whose metabolic pathway is under investigation, are incubated separately with the various postulated intermediates (now being used as substrates), the rate of oxygen uptake being measured manometrically. The absence of a lag period indicates compliance with the criterion of sequential induction. The presence of lag period, however small, is taken to mean that the induced enzymes were not originally present in the cells, the delay representing the time required for their synthesis.

The isolation of specific microbes with the required properties, for example , ability to grow freely as a pure culture in a simple salt mixture containing an aromatic compound as sole carbon source, is usually accomplished by the elective culture method (Winogradsky, 1949).

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The soil or sewage bed is continuously perfused with the simple medium for a period of few days, in the presence of a suitable amount of aromatic compounds (0.01 - 0.1% w/v). Sometimes, it is helpful to estimate the concentration of the aromatic substrate in the primary mixed culture medium. This may stay constant for days, and nothing seems to be happening at first but suddenly its disappearance starts, and the entire amount may be gone in a few hours. When active metabolism of the substrate is in progress , subcultures are made into simple media, thus causing preferential growth of a certain type of organism , ultimately leading to a predominance of the condi - tionally fittest. From such enrichment cultures, after plating out on simple media (stiffened with either agar or silica gel), the desired organism is isolated as a pure strain by the traditional methods of the microbiology.

Metabolic Pathways for Utilization of Aromatic Compounds:

It is generally accepted that dihydroxylation is a prerequisite for enzymatic fission of the benzene

ring (Mason, 1957). The hydroxyl groups may be ortho to each other, as in catechol and protocatechuic acid or para to each other as in gentisic and homogentisic acids. Catechol and protocatechuic acid have been shown to be the substrates for ring fission in the microbial degradation of many different aromatic compounds.

Although catechol is an intermediate during the degradation of polynuclear aromatic hydrocarbons, naphthalene, anthracene and phenanthrene by micro - organisms , a dihydroxylated polyaromatic compound is usually the first substrate for the ring fission.

A substituted aromatic nucleus presents microorganism with a choice as to their mode of attack. Results obtained in the microbial degradation of phenyl substituted acids by a Nocardia sp. indicate that the acid side chain is metabolized by a beta oxidation process, that is, the microorganism removes two carbon atoms at a time (Webley et al., 1962). Thus side chains with an odd number of carbon atoms are meta - bolized to benzoic acid, which is converted to catechol prior to ring fission. Phenyl substituted acids that contain an even number of carbon atoms are metabolized through phenyl acetic acid. Further metabolism of

phenyl acetic acid may proceed through either homogentisic acid (Kluyver et al., 1951 & Dagley et al.,1953) or homoprotocatechuic acid (Kumita, 1955/56, Dagley et al.,1962) depending on the species of microorganisms.

The enzymes catalyzing the hydroxylation of the aromatic ring have been termed mixed-function oxidases (Mason et al.,1955). In such reaction one atom of oxygen is incorporated into the substrate molecule. In the presence of a suitable electron donor the other oxygen atom is reduced to water. A typical reaction may be represented by the euation :

$$R — H + O_2 + XH_2 — ROH + H_2O + X$$

Where RH represents the substrate molecule and XH_2 represents the electron donor.

Microorganisms apparently utilize a variety of electron donating compounds in hydroxylation reactions. Salicylate hydroxylase, the enzyme that converts salicylic acid to catechol, has been purified from cells of a Pseudomonas sp. (Katagiri et al.,1966). The enzyme contains one mole of flavin adeninedinucleotide (FAD) per mole of enzyme protein. Since the reaction also requires reduced nicotinamide adeninedinucleotide ($NADH_2$)

and oxygen, the mechanism is pictured as a reduction of an enzyme - FAD - salicylate complex by $NADH_2$. Subsequent oxygen fixation by the complex results in the liberation of carbon dioxide with the formation of catechol and the oxidized enzyme.

The enzyme that forms protocatechuic acid from p-hydroxy benzoic acid also contains one mole of FAD per mole of enzyme protein (Hosokawa and Stanier, 1966). In contrast to salicylate hydroxylase, this enzyme requires reduced nicotinamide adeninedinucleotide phosphate ($NADPH_2$) for activity.

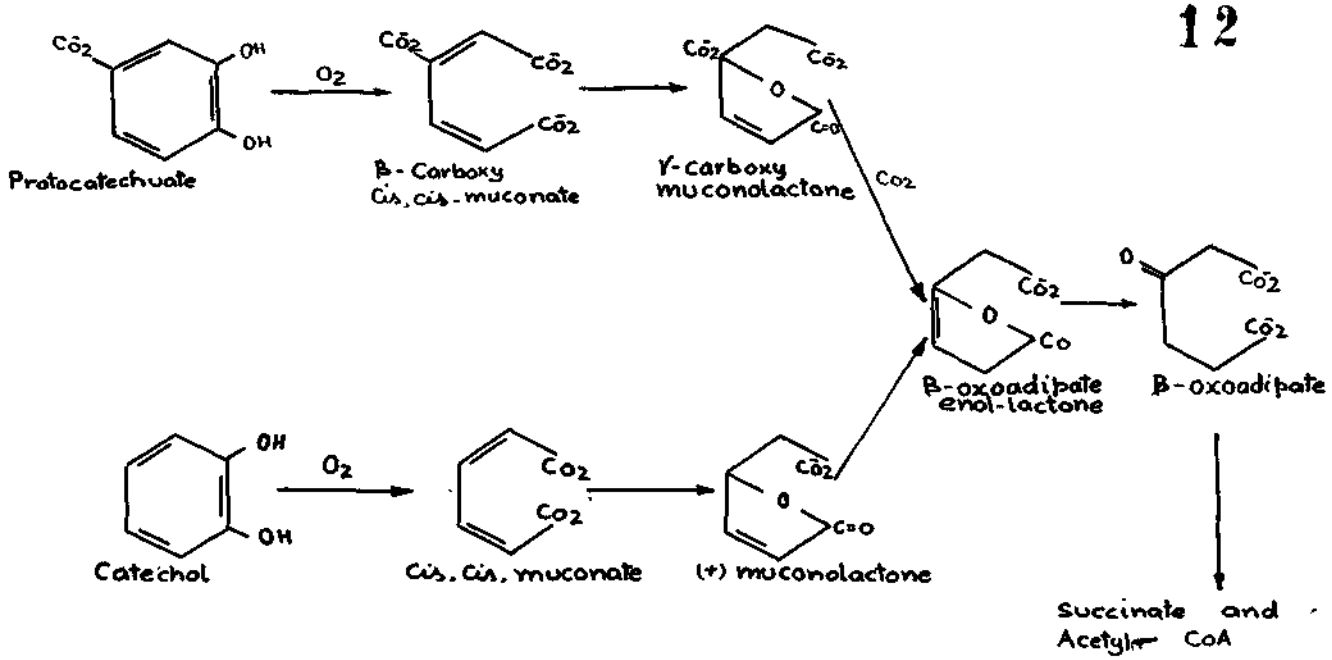
Phenyl alanine hydroxylation is catalyzed by an enzyme present in cells of a Pseudomonas sp. Enzymatic activity is dependent on the presence of dimethyltetrahydropteridine and $NADH_2$ (Guroff and Ito, 1965). Non-heme protein participates in the hydroxylation of octane (Peterson et al., 1966) , camphor (Gunsalus et al., 1967) and steroid (Kimura and Suzuki, 1967). These low molecular weight proteins take part in the transfer of electrons from $NADH_2$ to the enzymes catalyzing oxygen fixation into the substrate molecule. Since few enzymes which

hydroxylate the aromatic molecules have been purified, it seems possible that non-heme protein participates in the hydroxylation of aromatic compounds.

Catechol and protocatechuic acid are metabolic intermediates in the microbial degradation of many aromatic compounds. Consequently, these dihydroxylated benzene derivatives have been the objects of intensive investigation. Hayaishi and Hashimoto (1950) isolated an enzyme, catechol-1,2-dioxygenase that catalyzed the incorporation of molecular oxygen into the catechol molecule. The reaction product was identified as cis, cis-muconic acid. An analogous reaction due to protocatechuic acid -3,4-dioxygenase was reported for the metabolism of protocatechuic acid which resulted in the formation of β -carboxy cis, cis-muconic acid (Stanier and Ingraham, 1954). Both dioxygenases have been purified and each contains Fe^{+++} at its active centre (Gibson, 1968).

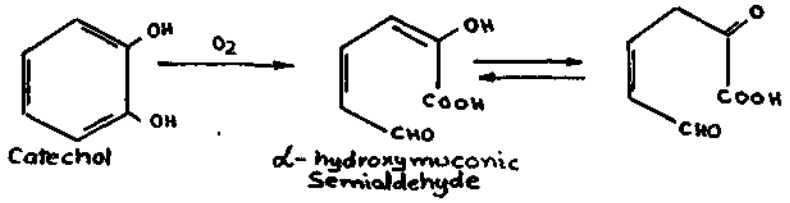
An alternative enzymatic cleavage of catechol was reported by Dagley and Stopher (1959). The enzyme is catechol-2,3-dioxygenase and the product is α -hydroxy-muconic semialdehyde. Dagley et al., (1960) also reported

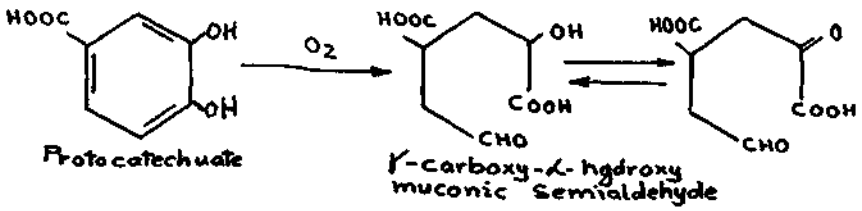
similar type of reaction for protocatechuic acid. This enzyme , protocatechuic acid-4,5-dioxygenase, oxidized protocatechuic acid to α -hydroxy - γ - carboxy muconic semialdehyde. The enzyme is extremely sensitive to oxygen and is easily inactivated in the presence of air. Crystallised 2,3-dioxygenase contains Fe^{++} at its active centre. Ornston and Stanier (1966) have reinvestigated the metabolism of these ring fission products. Their studies with Pseudomonas putida showed that the common intermediate, in the sequence of reactions initiated by catechol -1,2-dioxygenase and protocatechuic acid-3,4-dioxygenase was β-oxoadipic acid enol-lactone. In addition, a new intermediate compound, γ -carboxy muconolactone (γ -carboxy - γ - carboxy methyl- Δ^{β} - butenolide) was identified on the catabolic pathway of protocatechuic acid. It is now established that the three reactions responsible for the conversion of protocatechuic acid to β-oxoadipic acid enol-lactone are analogous to the reactions utilized to convert catechol to β-oxoadipic acid enol-lactone.



Degradation of Catechol and Protocatechuate by Pseudomonas putida.

The reaction sequences catalyzed by catechol-2,3-dioxygenase and protocatechuic acid-4,5-dioxygenase led to the formation of highly reactive carbonyl compounds.





The further metabolism of ring fission compound results in the formation of 2-oxo-4-hydroxyvaleric and formic acids. The former subsequently undergoes aldol cleavage to form pyruvic acid and acetaldehyde (Dagley and Gibson , 1965).

Scope of the thesis :- The material presented within this work is concerned with the study of bacterial degradation of benzaldehyde. Benzaldehyde occurs in the bitter almonds (Dhingra and Shukla, 1947), Pectis pappose (Bradley and Haagen, 1949), Anise and fennel oils (Monod and Dorton, 1950). It is also reported in the male sex hormone of Apamea monoglypha , Leucania impura and Phlogophora meticulosa (Aplin & Birch, 1968). In the sub-tropical fruit plants, Passiflora quadrangularis, and Bignonia venusta, it is known to stimulate the root formation (Traub , 1938). Joshua and Skinner (1918) have reported that it reduces the yield of wheat crop. The effect was very marked when the concentration of nitrite was higher than the potassium ions and phosphorus pentoxide. It is believed

that benzaldehyde reduces the absorption of growth factors. Various workers have also reported its harmful effects on the animals. In vitro benzaldehyde has a local anaesthetic effect because it relaxes the tonus and inhibits the contraction of smooth muscle. It has an antiseptic effect on bacteria (Macht, 1919 ; 1922 ; Alves , 1940). The injections, of benzaldehyde in dogs and rabbits, decrease their blood pressure and respiration (Wingard et al., 1955 ; Romano et al., 1954). The urine of injected dog contained hippuric acid whereas ornithuric acid was isolated from the excreta of injected fowls (Crowdle & Sherwin, 1923 ; and Sherwin & Crowdle, 1922).

Benzaldehyde also has the antigenic properties. The horse serum, shaked with benzaldehyde, when injected to a group of rabbits, produced an antisera which gave precipitin reactions with normal and benzaldehyde treated horse serum. Some also gave reactions with normal sera from man and other common experimental species (Mutsaars and Robert, 1939). In the vapour phase as well as solution, it is toxic to invertebrates like Paramecia, nematodes, earthworms

and flies (Moore, 1917 ; Drabkin, 1953). It has been used as a repellent to honey bees (Papadopoulo, 1966). It is also used in vapour phase to control the molding in closed containers (Scheffer and Catherine, 1946 ; Galloway, 1952).

A 80-90 fold purification, of benzaldehyde dehydrogenase, has been achieved from the strain of Pseudomonas fluorescens (Stachow et al. , 1967). The human liver aldehyde oxidase was strongly inhibited when benzaldehyde was used as the substrate (Johns, 1967) but from the pig liver, the aldehyde oxidase has been purified by using benzaldehyde as the substrate (Ando, 1966). Benzaldehyde does not inhibit benzoic acid oxidase. However, its derivatives, such as benzaldehyde thiosemicarbazone, inhibited the activity of the enzyme (Tomcsanyi et al. , 1958). Benzaldehyde competitively inhibited glutamic dehydrogenase, by competing both with the substrate and NAD^+ (Yoshida, 1959).

It is apparent from the literature survey that no serious attempt has yet been made to study the bacterial pathway for the degradation of benzaldehyde.

It is not yet clear in what way the benzaldehyde using organism would oxidatively cleave its aromatic ring. To throw more light on this aspect an Achromobacter sp., capable of utilizing benzaldehyde as sole source of carbon, has been isolated from the soil. This work deals with the studies on the bacterial degradation of benzaldehyde. The data presented provide sufficient clue to the reaction sequence through which benzaldehyde and related compounds are utilized by the organism for the diverse processes associated with their metabolism.

V. EXPERIMENTAL

1 - M A T E R I A L S

Chemicals - Technical Benzaldehyde of BUSH was purified and stabilized by hydroquinone as described (Vogel, 1959). The following chemicals were obtained commercially and used without further purification:

NAD^+, $NADP^+$, puromycin dihydrochloride (Sigma chemical Company , U.S.A.); NADH , NADPH , O-nitrobenzoic acid (BDH, England); Chloramphenicol (Parke, Davis & Co Ltd.) ; crystalline bovine serum albumin (Mann Research Laboratories, U.S.A.);standard bacteriological media (Difco Laboratories, Michigan); silica gel G (E. Merck, Germany).

Special reagents and chemicals were prepared as described.

Peroxide-free ether - One litre of ether was vigorously shaken in a separating funnel with 20 ml of conc.ferrous sulphate solution (to 60 gm of crystalline ferrous sulphate was added 6.0 ml of conc. sulphuric acid and 110 ml of water). The ether layer

was separated and anhydrous calcium chloride added. After 24 hours, the ether was filtered, and kept overnight over sodium wire (Vogel, 1959).

To test that the ether thus obtained is peroxide-free, a small volume of ether was shaken with an equal volume of 2% potassium iodide and few drops of dilute hydrochloric acid. The appearance of blue colour on addition of a few drops of 1% (w/v) starch solution indicates the contamination of ether with peroxide (Vogel, 1959).

Alcohol - For the colorimetric estimation of benzaldehyde, it was found necessary to remove the traces of aldehyde impurities from the alcohol used for preparation of 1.5 N NaOH . The alcohol was kept overnight on KOH pellets (500 gm / lit) and refluxed for six hours before distillation.

2,4-Dinitrophenyl hydrazone - The 2,4-dinitrophenylhydrozone of benzaldehyde was prepared as described (Shriner et al., 1960). 0.4 gm of 2:4-dinitrophenyl -

hydrazine was added to 2 ml of conc. sulphuric acid and 3 ml water was added dropwise with stirring until solution was complete. To this warm solution was added 10 ml of 95% ethanol and 0.5 ml benzaldehyde dissolved in 20 ml of 95% ethanol. The resulting mixture was allowed to stand for 30 minutes at room temperature. Crystallisation of 2,4-dinitrophenyl - hydrazone occured in about 15 minutes. The hydrazone was recrystallised from 95% ethanol (Observed m.p. = 236-237° (uncorrected) ; reported m.p. = 237° (uncorrected).

2 - ANALYTICAL METHODS

Benzaldehyde 2:4-dinitrophenyl hydrazone - To quantitate the amount of benzaldehyde taken for various experiments, the prepared benzaldehyde solution was assayed by the method of Friedmann & Haugen (1943). 14.3 mg of the prepared hydrazone of benzaldehyde dissolved in 3 ml of ethyl acetate and the final volume

made to 250 ml with 95% ethanol (0.2 µ moles hydrazone/ ml), was used as working standard. To an aliquot containing 0 - 0.3 µ moles of hydrazone in 2 ml of 95% ethanol was added 5 ml of 1.5 N NaOH. After 15 minutes the optical density was recorded against the reagent blank in an AIMIL Biochem Absorptiometer using No.52 filter (500 - 540 mµ) (Fig. 1).

Proteins - Bovine serum albumin solution was used as standard protein. It was estimated by the method of Lowry et al.,(1951). To 1 ml of sample containing upto 180 µg protein was added 5 ml of alkaline copper reagent (comprising of equal volumes of 8% sodium carbonate and a solution of 0.3 gm copper sulphate, 0.6 gm Rochelle salt in a total volume of 500 ml). After 10 minutes at room temperature, 0.5 ml of diluted Folin reagent (1 N) was added. The colour was read after 30 minutes in AIMIL Biochem Absorptiometer using filter No.70 (630 mµ - 750 mµ) against the reagent blank prepared similarly except that water replaced the protein solution (Fig. 2).

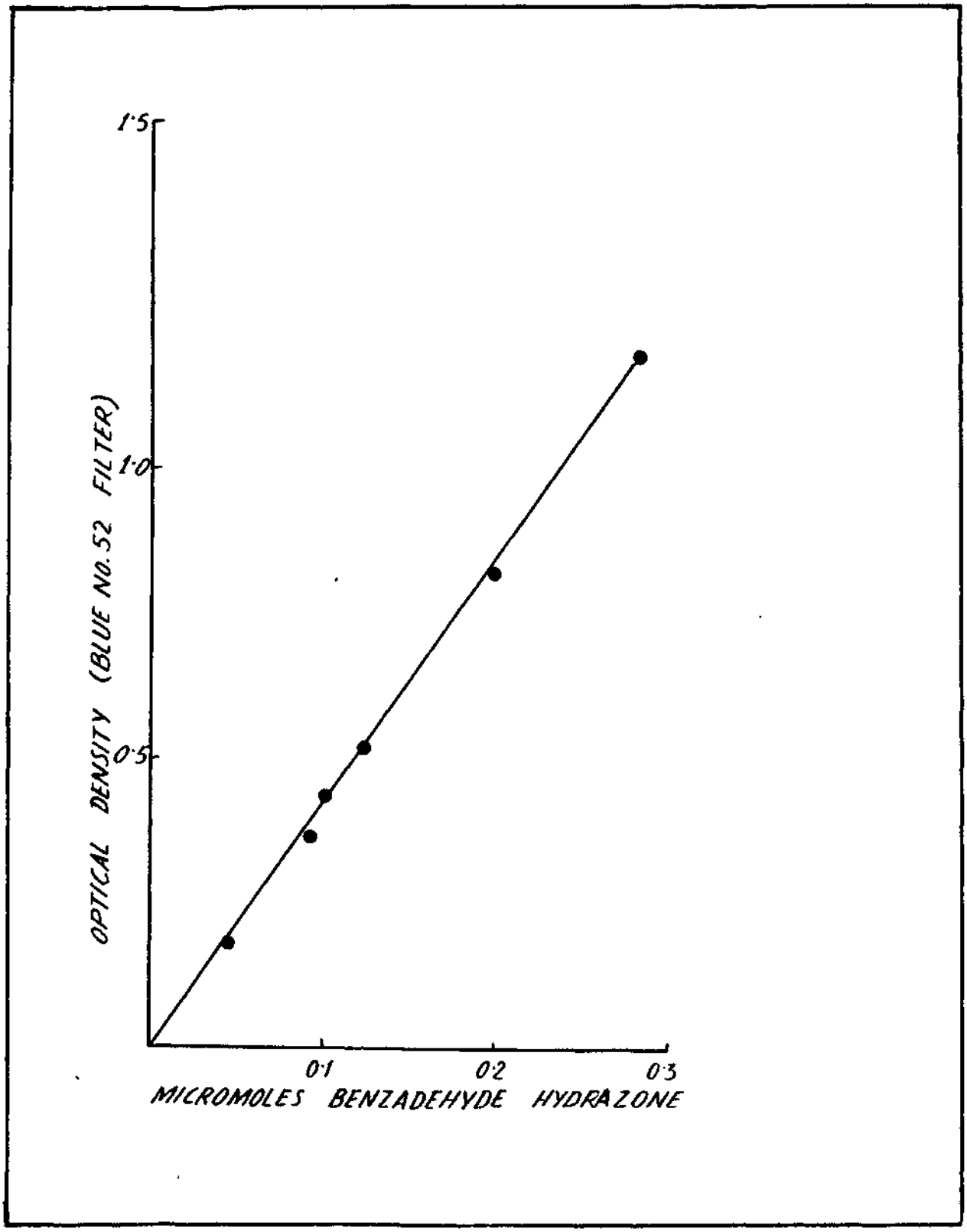
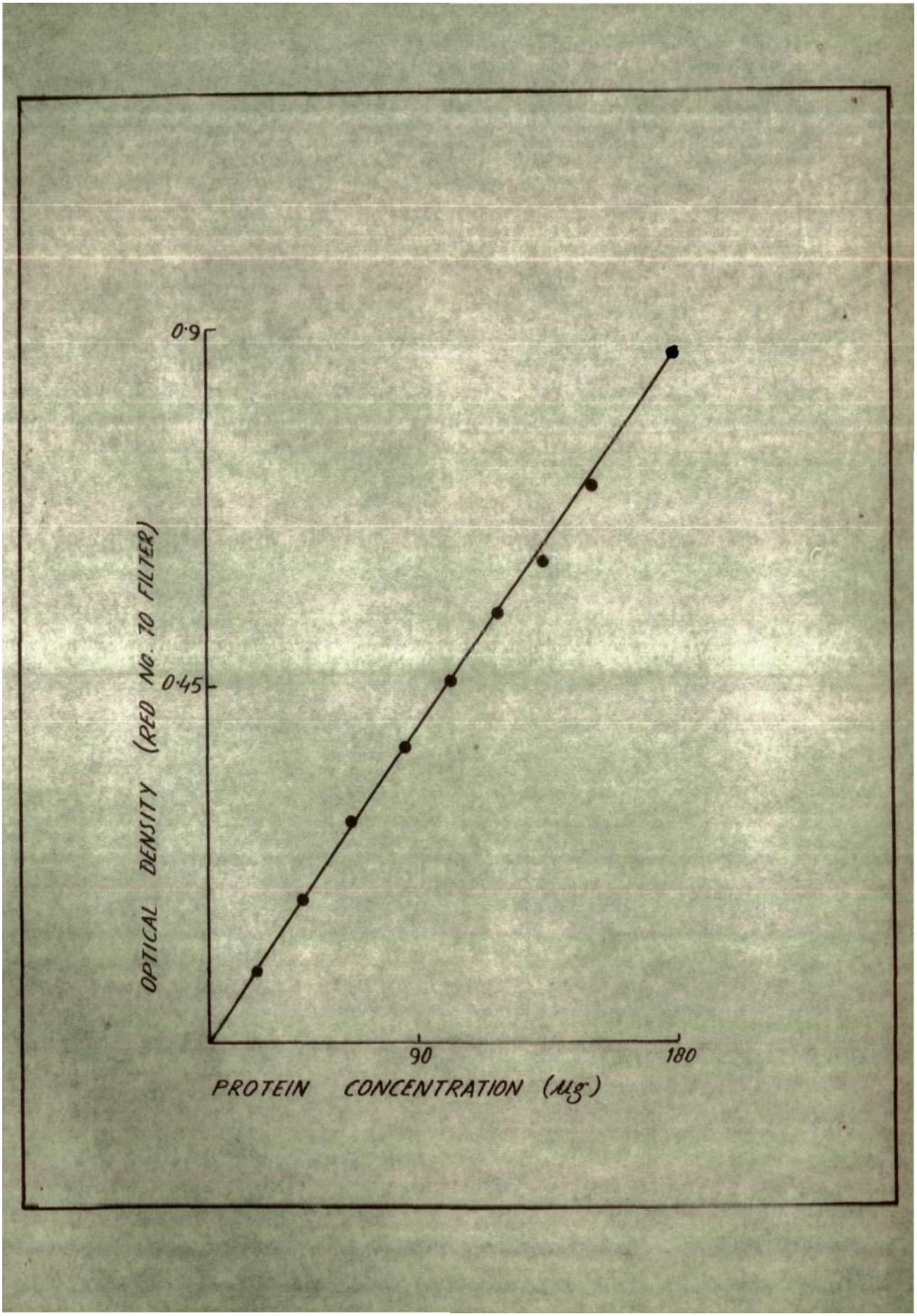

1·5
1·0
0·5
OPTICAL DENSITY (BLUE NO. 52 FILTER)
0·1
0·2
0·3
MICROMOLES BENZADEHYDE HYDRAZONE

Fig.2. Standard curve for protein determination. See text for details.


0·9
0·45
OPTICAL DENSITY (RED No. 70 FILTER)
90
180
PROTEIN CONCENTRATION (µg)

β-Oxoadipate - It was estimated by the catalytic decarboxylation method of Sistron and Stanier (1953). 0.1M 4-aminoantipyrin was prepared in 0.1M acetate buffer of pH 4.0. A suitable aliquot of the solution containing β-oxoadipate was taken in a Warburg flask to which was tipped 0.1 ml acetic acid and 0.4 ml of 0.1M 4-aminoantipyrin from the side arm. The carbon dioxide liberated was measured by direct method of Warburg. To further confirm the presence of β-oxodipate, Rothera test was performed. The incubation mixture was saturated with ammonium sulphate followed by 3 drops of concentrated ammonium hydroxide and few drops of 5% sodium nitroprusside. The development of the permanganate tinge is the indication of positive test (Hawk et al., 1948).

3 - CULTURE MEDIA

Each litre of mineral salt medium (Medium A) used for the isolation of the organism had the following composition:

K_2HPO_4	...	1.15 gm
NH_4NO_3	...	1.00 gm
KH_2PO_4	...	0.063gm
$MgSO_4.7H_2O$	...	0.04 gm
NaCl	...	0.02 gm
$FeSO_4.7H_2O$	...	0.02 gm
$MnSO_4$	...	0.02 gm
Soil Extract (50% w/v)	...	100 ml

The pH of the solution was 7 .

Each litre of mineral salt medium (Medium B) used for growth of the organism had the following composition and is similar to that used by Evans (1947).

$(NH_4)_2SO_4$	...	1.0 gm
$K_2H\ PO_4$	...	0.5 gm
$MgCO_3$	...	0.5 gm
$CaCl_2$	...	0.1 gm
NaCl	...	0.1 gm
$FeCl_3$	...	0.01gm
Soil Extract (50% w/v)	...	100 ml

The pH of the solution was 7.0

Initially when the cells were not fully adapted to benzaldehyde the addition of soil extract was found beneficial. However after complete adaptation it was not added as it had no effect on the growth of the organism.

4 - PREPARATION OF MEDIA FOR GROWTH

(i) Nutrient broth - It was prepared by dissolving 8 gm dehydrated nutrient broth (5 gm peptone and 3 gm beef extract) in 1000 ml of water.

(ii) Nutrient Agar - 2% agar was added to nutrient broth.

(iii) Benzaldehyde broth - 0.05% (v/v) Benzaldehyde was added to medium B.

(iv) Benzoate broth - 0.05% (w/v) benzoate was dissolved in the medium B.

(v) p-Hydroxybenzoate broth - 0.05% (w/v) p-hydroxybenzoate was dissolved in the medium B.

(vi) m-Hydroxy benzoate broth - 0.05% (w/v) m-hydroxy benzoate was added in the medium B.

(vii) Adipate broth - 0.05% (w/v) adipate was dissolved in the medium B.

(viii) Succinate broth - 0.05% (w/v) succinate was dissolved in the medium B.

(ix) Acetate broth - 0.05% (w/v) acetate was dissolved in the medium B.

(x) Benzaldehyde Agar - 2% agar was added to benzaldehyde broth.

(xi) Potato Agar - 100 gm of cleaned potato pieces were added to 300 ml water and steamed in an autoclave for one hour. The potato extract was filtered through cotton and diluted to one litre with distilled water. 2% agar was added.

(xii) Nutrient Gelatin - It was prepared by dissolving 120 gm of gelatin in one litre of nutrient broth.

(xiii) Glucose broth - 0.05% glucose (w/v) was dissolved in medium B.

5 - MAINTENANCE OF CULTURES

The stock cultures of the organism were maintained on nutrient-agar slants at 4^{0}. Fresh transfers

of the slants were made every fortnight. The organism could be kept for longer periods at 4°.

For the preparation of inoculum for metabolic studies, the organism was transferred from the nutrient-agar slants to the appropriate media, as indicated. However for identification purposes, the inoculum was prepared from nutrient-broth grown cells.

6 - IDENTIFICATION PRODEDURES

The staining procedures used for the identification of the organism have been described (Peltier et al., 1955). All additional media used in the identification of the organism were prepared as described in "Manual of Methods for Pure Culture Study of Bacteria" published by the Society of American Bacteriologists (1950).

7 - PREPARATION OF RESTING CELL SUSPENSIONS

The liquid cultures were grown at 30-35° with constant shaking. After 20 hours growth, the cells harvested in log-phase were washed thrice with double distilled water, suspended in the medium B and used for experimental purposes.

8 - PAPER CHROMATOGRAPHY

Routine descending chromatography procedures were used to detect the accumulated intermediates in the incubation mixtures using Whatman No.1 paper.

Solvents - Solvents were generally prepared just prior to use. In case of immiscible solvents, the organic layer was used as mobile phase. The chromatograms were conditioned prior to development. The following solvents were used.

ip : Am - Isoproponal:Ammonia (Sp.gravity 0.68): Water : : 200:10:20 (Stowe and Thimann , 1954).

BZ : Ac - Benzene : Acetic acid : Water : :
125 : 72 : 3
(Ivor Smith , 1958).

Bu : Py - n-Butanol : Pyridine : Water : :
140 : 30 : 30.
(Ivor Smith , 1958).

Bu : Am - n-Butanol : Ammonia : Water : :
70 : 20 : 10.
(Copius - Peere boom and Beekes, 1964).

Detection Reagents - After development the chromatograms were oven dried and the following reagents were used for localization of unknown compounds.

(i) Ultra - Violet light - The phenolic compounds were detected by their characteristic quenching effect when exposed to ultra violet light.

(ii) Sulphanilic Reagent - (a) Sulphanilic acid 9 gm, in 90 ml concentrated HCl and 900 ml water ... 2 Vol.
Sodium nitrite 5% (w/v) ... 1 Vol.
(b) Sodium carbonate anhydrous, 10% (w/v) in water ... 2 Vol.

When required, the reagent (a) was chilled in ice bath and solution (b) added slowly as the mixture effervesces vigorously.

(iii) Nitraniline Reagent - (a) p-nitraniline - 1.5 gm, in 45 ml concentrated HCl and 950 ml water ... 10 Vol. (b) Sodium nitrite, 5% (w/v) in water ... 0.2 Vol.
(c) Sodium carbonate anhydrous, 10% (w/v) in water ... 10 Vol. When required, the reagent was prepared by adding nitrite to the nitraniline and then adding the carbonate.

(iv) Bromophenol Blue - Methyl Red

(a) Bromophenol Blue was prepared by dissolving 120 mg in 100 ml of water.
(b) 60 mg of Methyl Red was dissolved in 100 ml of ethanol.
Solutions (a) and (b) were mixed and 100 ml of potassium phosphate buffer, pH 7.2 added. Benzoic acid spots were not very clear with it. To make the spots more prominent 0.5 gm potassium permanganate and 1 gm sodium carbonate was added to the mixed reagent.

Rf values - Standard aromatic compounds were applied together with the sample. The Rf values of compounds of interest to this work, run in various solvents are summarized in Table 1.

9 - THIN LAYER CHROMATOGRAPHY

To 40 gm of silica gel G was added 90 ml of water to make a thick slurry. The glass plates (20 x 20 cm) were coated to a thickness of 0.5 mm with a commercial applicator. The plates were air dried for 2 hours, activated at 105° for half an hour and allowed to cool in a dessicator. Samples were applied along a line 2 cm from the end and dipped in the developing solvent.

Solvents - The following solvents were generally prepared just prior to use.

Bz : Ox - Benzene : Dioxane : Acetic acid : :
90 : 25 : 4 (Pastuska , 1961).

TABLE - I

Rf values of aromatic compounds by paper chromatography.

COMPOUND	SOLVENTS			COLOUR OF THE SPOT DETECTION REAGENTS		
	Ip:Am	Bu:Py	Bu:Am	Nitra-nilii-ne	Sul-phani-lic acid	Bromo-thymol Blue-Methyl Red.
m-Hydroxy benzoic acid	0.32	0.31	-	Brown	Yellow to orange	
Salicylic acid	-	0.46	-	Orange	-	
p-Hydroxy benzoic acid	0.23	0.32	-	Reddi-sh Brown	Brown to Orange	
Protocate - chuic acid	0.06	0.12	-	Brown	Light brown	
Catechol	0.77	0.90	-	Vio-let	Brown	
Benzoic acid	-	-	0.48	-	-	Pink

ip : Am - Isopropylalcohol : Ammonia (sp.gravity 0.88) : Water : : 200 : 10 : 20.
(Stowe and Thimann, 1954).

Detection Reagents - After development the plates were oven dried and the following reagents were used for localization of unknown phenolic compounds.

(i) Sulphanilic Reagent - It was prepared as described for paper chromatography.

(ii) Tetrazotized Benzidine - (a) 1.5 gm benzidine was dissolved in 14 ml concentrated HCl and diluted with water to one litre.
(b) Aqueous 10% (w/v) sodium nitrite solution .
When required equal volumes of solution (a) was mixed with the solution of (b) (Koch and Krieg , 1938).

Rf values - The Rf values of the compound of interest to this work are summarized in the Table II .

TABLE - II

Rf values of phenolic compounds by thin layer chromatography

COMPOUND	SOLVENTS		COLOUR OF THE SPOT DETECTION REAGENTS	
	ip:Am	Bz:Ox	Tetrazotized benzidine	Sulphanilic acid
m-Hydroxy benzoic acid	0.40	0.61	Yellow	Yellow
p-Hydroxy benzoic acid	0.36	0.59	Yellowish brown	Yellow
Protocatechuic acid	0.037	0.54	Light brown	Brown
Catechol	0.61	0.71	Dark brown	Dark brown
2,4-dihydroxy benzoic acid	-	0.68	Greyish brown	-

10 - MANOMETRIC METHODS

The ability of resting cell suspension of the *Achromobacter sp.* to oxidize a number of substrates was determined by conventional manometric technique (Umbriet *et al.*, 1957). Although it is not normal practice, the respiration was measured in medium B containing various carbon sources as it was anticipated that the pattern of enzyme induction would be of as much interest as the initial rates of oxygen uptake. It is usually difficult to avoid some enzyme induction, unless protein synthesis inhibitors are used. Suspensions of bacteria, washed thrice with ice cold double distilled water were invariably used within one hour of harvesting. Each 15 ml Warburg vessel, contained bacterial suspension in 1.6 ml medium B. The reaction was started by tipping 0.2 ml of 10 mM substrate dissolved in water (pH 7.0). The central well contained 0.2 ml of 20% KOH with a small roll of filter paper. The reaction was run at 30° with air as gas phase.

VI. R E S U L T S

1 - ISOLATION OF ORGANISM BY THE ENRICHMENT CULTURE TECHNIQUE

The organism was isolated from the local garden soil. Due to bacteriostatic effect of benzaldehyde, initial attempts to isolate a benzaldehyde utilizing organism were unsuccessful. To overcome this difficulty, a drop of benzaldehyde was first added to the medium A and its concentration was gradually increased to 0.05% (v/v). Approximately 10 gm soil was added to 25 ml of the medium A containing 0.05% (v/v) benzaldehyde and incubated at room temperature. The growth was observed after two days. After five successive transfers in the same medium, the organism was plated on the benzaldehyde-agar. A colony of the rapidly growing organism was selected and replated to yield second plates containing a single colony type. The pure cultures thus obtained were transferred to the nutrient-agar slants and stored at 4°.

2 - DETERMINATION OF CELL YIELD - Cell yields were determined turbidimetrically in Klett-Summerson Photometer using green filter No.54. Concentrations

expressed in mg per litre represent mg dry cell equivalent per litre. A standard curve was prepared from readings of appropriate dilutions of a salt free suspension of the organism (Fig. 3).

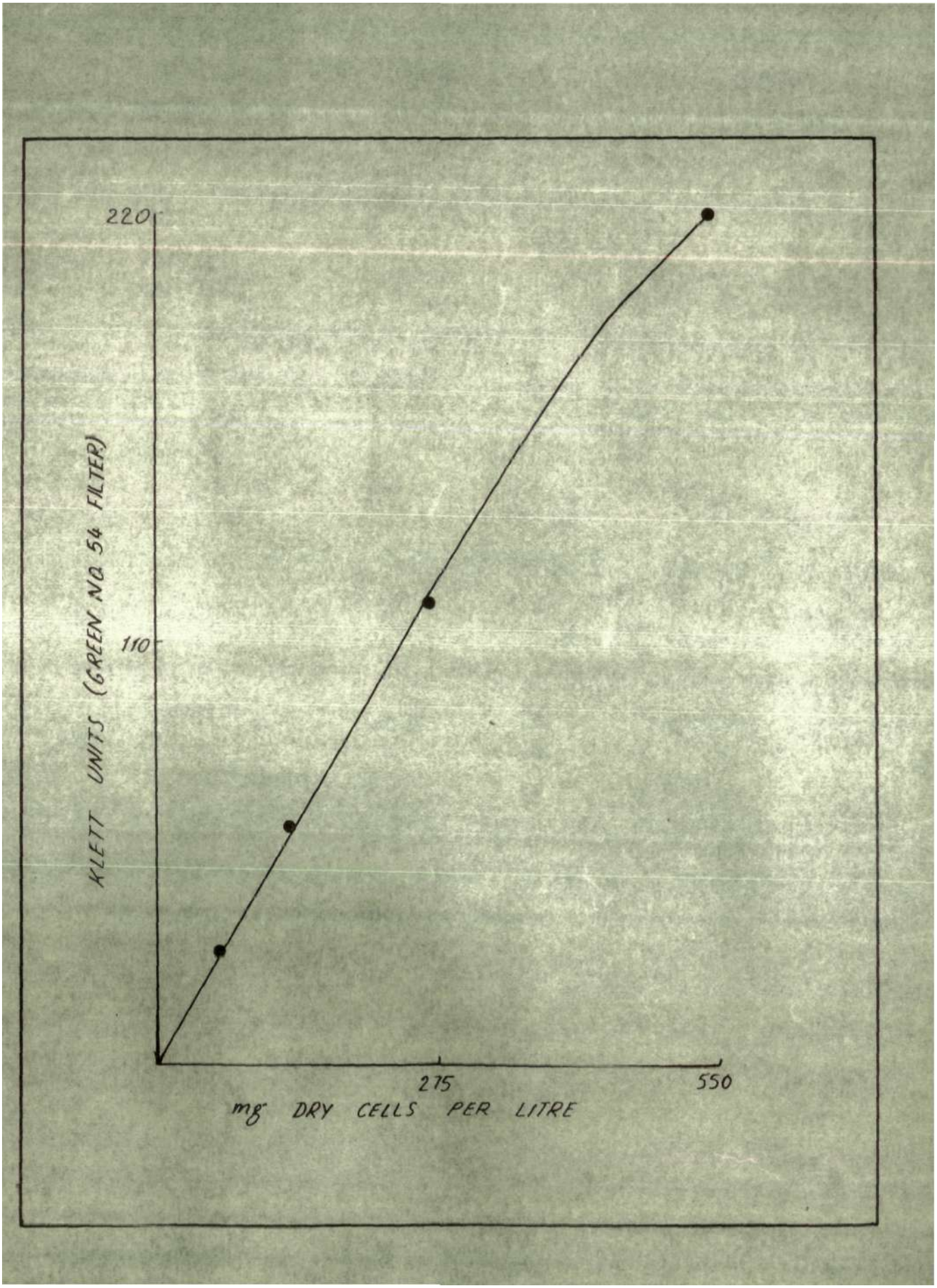
The cells were grown in benzaldehyde broth for 24 hours, harvested, washed thrice with glass distilled water and suspended again in water and used. Suitable aliquots of this suspension were dried at 110° for 12 hours to determine the relation between Klett units and dry cell equivalent.

3 - IDENTIFICATION OF THE ORGANISM

A. Morphological Characteristics - The organism is gram negative, non-acid fast, small rods of 2.5 to 3 μ occuring in chains of 2 to 3. The capsule formation could only be observed after twenty four hours growth. The bacterium is motile and has peritrichous flagella. There was no endospore formation.

Surface colonies in nutrient-agar, benzaldehyde-agar and potato-agar were smooth, circular,

Fig.3. Relationship between turbidity and dry cell weight.


220
110
KLETT UNITS (GREEN No 54 FILTER)
275
550
mg DRY CELLS PER LITRE

convex, translucent and glistening with wrinkled margins. The slants on nutrient-agar as well as in benzaldehyde-agar showed moderate beaded growth without any change in the color or odor of the medium.

As compared to the nutrient broth the organism showed less growth in benzaldehyde-broth and produced brown colour and black particles which were deposited on the sides of the culture flasks. No pellicle formation was observed.

B. Nutritional Characteristics

(1) Replacement of Carbon Sources - Various carbon sources examined in the concentrations of 0.01 - 0.05% (w/v) for their ability to replace benzaldehyde as sole source of carbon, included benzoate, p-hydroxy benzoate, m-hydroxy benzoate, 2,4-dihydroxy benzoate, 2,4-dihydroxy benzaldehyde, salicylaldehyde, salicylate, acetate, citrate and succinate. The results are shown in Table III. It is apparent that all the compounds supported the growth of the organism. However the growth was poor on the 2,4-dihydroxy benzoate

TABLE - III

Effect of Various Carbon Sources on the Molar Growth Yield

250 ml Erlenmeyer flasks containing 25 ml sterile medium B and appropriate compounds at the concentration levels of 2.5, 7.5, 10, & 12.5 mg were inoculated in an identical manner with 0.13 mg dry cell equivalent of benzaldehyde-grown cells. The cultures were shaken for four days at 30°. The turbidity was measured as described under methods. The 'Molar Growth Yield' was calculated by the linear portion of the graph of the growth of the organism plotted against substrate concentration (Dawes, 1962).

SUBSTRATE		MOLAR GROWTH YIELD (µg dry wt/ µ mole substrate.)
Benzaldehyde	...	56
Benzoate	...	56
p-hyd.benzoate	...	56
Adipate	...	50
Acetate	...	42
2,4-dihydroxy benzaldehyde	...	40
Citrate	...	40
Succinate	...	40
2,4-dihydroxy benzoate	...	30
m-hydroxy benzoate	...	26
Salicylaldehyde	...	20
Salicylate	...	17

and m-hydroxy benzoate. It was almost negligible on salicylaldehyde and salicylate.

(ii) Replacement of Nitrogen Sources - Ammonium sulphate, Ammonium chloride, Ammonium nitrate, Ammonium dihydrogen phosphate and peptone in the concentration of 0.1% were examined for their ability to replace ammonium sulphate as the sole nitrogen source. Each of these compound was suitable nitrogen source (Table IV). Comparatively, peptone, ammonium sulphate and ammonium chloride were better nitrogen sources.

C. Biochemical Characteristics

(i) Action on Carbohydrates - Nutrient broth supplemented with bromocresol - purple indicator was used to determine carbohydrate dissimilation properties of the organism. Neither acid nor gas was produced from any of the carbohydrates used, which included, glucose, galactose, mannose, rhamnose, xylose, fructose, lactose, sucrose, maltose, mannitol and glycerol.

T A B L E - IV

Effect of Nitrogen Sources on the Total Cell Yield.

In a total volume of 10 ml sterile medium B containing no ammonium sulphate but 10 mg of indicated nitrogen sources and 0.05% (v/v) benzaldehyde were aseptically inoculated with 0.135 mg dry cell equivalent of benzaldehyde-grown cells. The cultures were incubated at 30°. The 'Total Cell Yield' was calculated from turbidity measurements as described earlier.

COMPOUND	TOTAL CELL YIELD	
	24 hours	48 hours
	(mg dry cell / L)	
Peptone	85.0	125.0
Ammonium sulphate	52.0	112.0
Ammonium chloride	51.0	110.0
Ammonium nitrate	41.0	87.5
Ammonium dihydrogen phosphate	37.0	80.0

(ii) Gelatin Liquefication - In nutrient gelatin stab at 20°, the growth of the organism was in beaded form. On third day of inoculation, gelatin slowly liquefied (Saccate). The organism grew best on the top of the line of puncture and did not produce pigment.

(iii) Action on Litmus Milk - The organism grew well in litmus milk. After 24 hours of growth there was neither change of litmus colour nor the milk coagulated.

(iv) Reduction of Nitrates - The organism reduced nitrates without the production of ammonia.

(v) Miscellaneous Characteristics - The organism was incapable of producing acid, acetyl methyl carbinol, hydrogen sulphide and indole in tryptone broth. It did not hydrolyze starch.

D. Factors Influencing the Growth of the Organism -

In an effort to find the optimum conditions for the growth of the organism the effect of various factors, on the growth of the organism was studied. Total cell

yield has been expressed in terms of mg dry cell equivalent per litre even though the incubation was carried out in smaller volumes.

The studies were carried out in 100 ml Erlenmeyer flasks fitted with a Klett-tube side arm to permit continuous evaluation of culture trubidity under sterile conditions.

(i) Effect of Temperature - As shown in the Table V, the optimal growth was observed over the range of 30-37°.

(ii) Effect of Hydrogen Ion Concentration - The growth of the organism was vigorous over the range of pH 5-7 with maximal growth at pH 7.0 (Table VI)

(iii) Effect of Shaking - It is apparent from the Table VII that the shaking increased the rate of growth as well as the total cell yield.

(iv) Effect of Inoculum Size - 100 ml flasks containing 10 ml of sterile benzaldehyde-broth, were inoculated with indicated concentrations of 24 hours benzaldehyde-broth grown cells. The culture flasks

TABLE - V

Effect of Temperature on the Total Cell Yield

100 ml Erlenmeyer flasks containing 10 ml sterile benzaldehyde broth were aseptically inoculated with 0.13 mg dry cell equivalent benzaldehyde-grown cells and incubated at indicated temperatures. After 24 hours of growth, 'Total Cell Yield' was calculated from turbidity measurements as described earlier.

TEMPERATURE		TOTAL CELL YIELD (mg dry cell /L)
10 - 15°	...	45
15 - 20°	...	65
20 - 25°	...	135
30°	...	130
30 - 37°	...	160

T A B L E - VI

Effect of Hydrogen Ion Concentration On Total Cell Yield

100 ml Erlenmeyer flasks containing 10 ml of sterile buffered nutrient broth at indicated pH were aseptically inoculated with 0.15 mg dry cell equivalent of benzaldehyde-grown cells. The 'Total Cell Yield' was calculated from turbidity measurements as described earlier.

pH		TOTAL CELL YIELD 24 hours	48 hours
		(mg dry cell /L)	
5	...	150	410
6	...	407	410
7	...	420	430
8	...	325	325
9	...	135	162
10	...	155	165

TABLE - VII

Effect of Shaking On Total Cell Yield

100 ml Erlenmeyer flasks containing 10 ml of sterlized benzaldehyde broth were aseptically inoculated with 0.245 mg of dry cell equivalent. At the indicated time intervals the 'Total Cell Yield' was calculated from turbidity measurements.

TIME (Hours)		TOTAL CELL YIELD	
		No Shaking	Shaking
		(mg dry cells/L)	
0	...	25.0	25.0
1	....	35.0	40.0
3	...	52.5	55.0
9	...	60.0	90.0
14	...	62.5	110.0

were incubated at 30° on rotary shaker. It is apparent from the growth curves (Fig.4) that higher inoculum concentrations although increase the rate of growth but the total cell yield finally becomes constant.

(v) Effect of Benzaldehyde Concentration - Table VIII shows the effect of benzaldehyde concentration on the total cell yield. It is clear that beyond 0.06% (v/v) of benzaldehyde concentration the cell yield: benzaldehyde ratio decreases. Therefore for all the studies in benzaldehyde-broth the substrate level used was 0.05% (v/v).

(vi) Growth Rates of the Organism with Different Substrates - The growth rates for batch cultures of the bacterium grown on benzaldehyde and related compounds are shown in Table IX. The growth rates with catechol were not determined because of the brown colour produced in media containing catechol. Appreciable time lags (more than 30 minutes) before the onset of exponential growth were observed, with substrates as indicated. Calculations of the length of

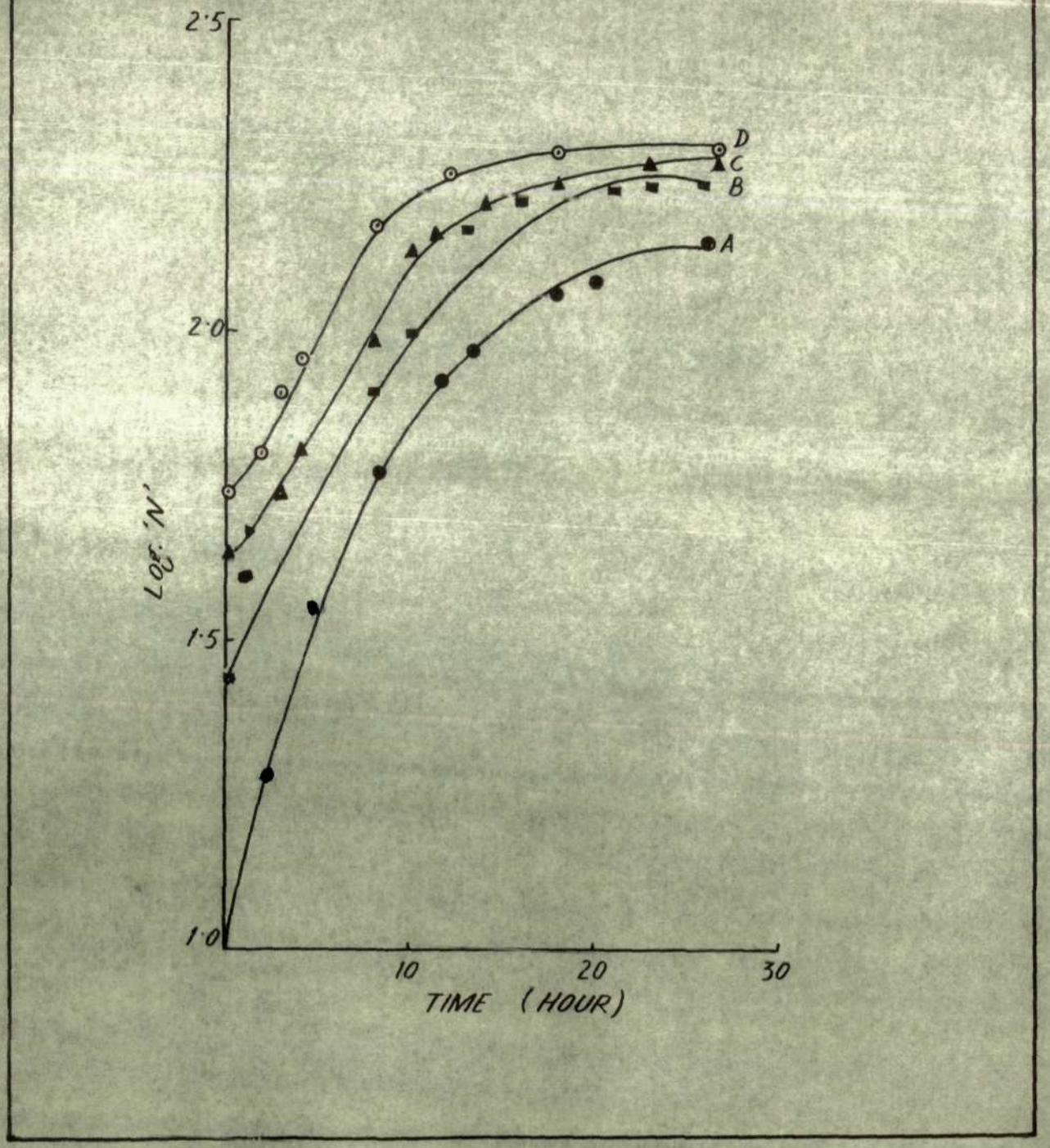
Fig.4. Effect of inoculum size on growth. At different time intervals the cell yield was calculated from turbidity measurements. Log 'N' represents log of mg dry cells per litre. See text for details.

A ... 0.100 mg dry cell equivalent/flask

B ... 0.275 mg dry cell equivalent/flask

C ... 0.425 mg dry cell equivalent/flask

D ... 0.550 mg dry cell equivalent/flask


2·5
2·0
1·5
1·0
LOG 'N'
D
C
B
A
10
20
30
TIME (HOUR)

T A B L E - VIII

Effect of Benzaldehyde Concentration On Total Cell Yield

100 ml Erlenmeyer flasks containing varying concentration of benzaldehyde in 10 ml of medium B were aseptically inoculated with 0.13 mg dry cells. The cultures were incubated at 30°. After 24 hours cell yield was calculated from turbidity measurements as described earlier.

BENZALDEHYDE (ml / L)		TOTAL CELL YIELD (mg dry cell/L)	YIELD (mg dry cells/ml benzaldehyde.)
0.3	...	85	283
0.4	...	115	288
0.5	...	110	220
0.6	...	115	192
0.7	...	42.5	61

TABLE - IX

Growth Rates with different substrates

100 ml Erlenmeyer flasks containing 10 ml sterile medium B and appropriate compounds were aseptically inoculated with 0.13 mg dry cell equivalent of benzaldehyde-grown cells. The cultures were incubated at 30° and growth was followed by measuring turbidity at appropriate time intervals. Results are means of two experiments. Lags are described as present or absent.

SUBSTRATE	SUBSTRATE CONCENTRATION		LAG
	1mM	1.5mM	
	Mean generation time (minutes)		
Benzaldehyde ..	35	40	-
Benzoate ..	40	38	-
p-hydroxy-benzoate ..	70	57	+*
2,4-dihydroxy benzaldehyde ..	58	58	+*
Salicylaldehyde ..	83	70	-
m-hydroxy benzoate	148	120	+
2,4-dihydroxy benzoate ..	104	90	-
Adipate ..	33	90	+
Glucose ..	148	120	+
Citrate ..	164	181	+
Acetate ..	168	181	+*
Succinate ..	289	310	+

* Lag was observed at higher concentration only.

these lags by the conventional methods (e.g.Dawes, 1962) gave values upto 60 minutes.

4 - CLASSIFICATION OF THE ORGANISM - By virtue of the characters listed above the organism has been identified as a member of the genus "Achromobacter".

5 - METABOLIC STUDIES

A. Manometric studies - Manometric experiments were carried out in a conventional Warburg apparatus using single arm flask shaken at 105 strokes per minute (Umbriet et al., 1957). The organism grown in medium B supplemented with indicated carbon sources (0.05% w/v or 0.05% v/v) were harvested in log phase by centrifugation at 4°. The bacterial suspensions washed thrice with ice-cold double distilled water were invariably used within one hour of harvesting . In a total volume of 2 ml each 15 ml Warburg flask contained bacterial suspensions in 1.6 ml medium B. The reaction was started by tipping 0.2 ml of 10 mM substrate (dissolved in water and adjusted to pH 7.0 with 0.1 N NaOH if necessary) from the side arm. For oxygen uptake studies the central well contained 0.2 ml of 20% KOH and a small roll of filter paper. The reaction was run at 30° in air. A preliminary experiment showed that the rate of oxygen uptake was proportional to the amount of bacteria at least upto the concentrations used.

For following carbon dioxide evolution KOH was omitted from the central well and the direct method of Warburg was used. To correct for endogenous oxygen uptake or carbon dioxide evolution, substrate was omitted from appropriate flasks. Unless mentioned otherwise the data obtained are average of duplicate experiments and have been corrected for endogenous gas exchange.

(i) Benzaldehyde-grown cells - As shown in Figs. 5, 6, 7 and 8 benzaldehyde-grown cells oxidized benzaldehyde, benzoate, protocatechuate, catechol, 2,4-dihydroxy benzaldehyde, salicylaldehyde, succinate, fumarate, malate and acetate without lag phase whereas p-hydroxy benzoate, m-hydroxy benzoate, adipate, citrate and α-ketoglutarate were oxidised with lag. The oxygen uptake for salicylaldehyde and 2,4-dihydroxy benzaldehyde was very poor. The organism did not oxidise salicylate, indicating that this compound may not be on the direct pathway of benzaldehyde catabolism. In presence of 0.3 mM puromycin, the rate as well as total oxygen uptake of benzaldehyde decreased (Fig. 9). The rate of oxidation of p-hydroxy benzoate and m-hydroxy benzoate

Fig.5. Ability of benzaldehyde-grown cells to oxidize benzoate, catechol, protocatechuate and acetate. Each Warburg flask contained 9 mg dry weight equivalent of benzaldehyde-grown cells and 2 μ mole substrate.

A ... Benzoate

B ... Catechol

C ... Protocatechuate

D ... Acetate

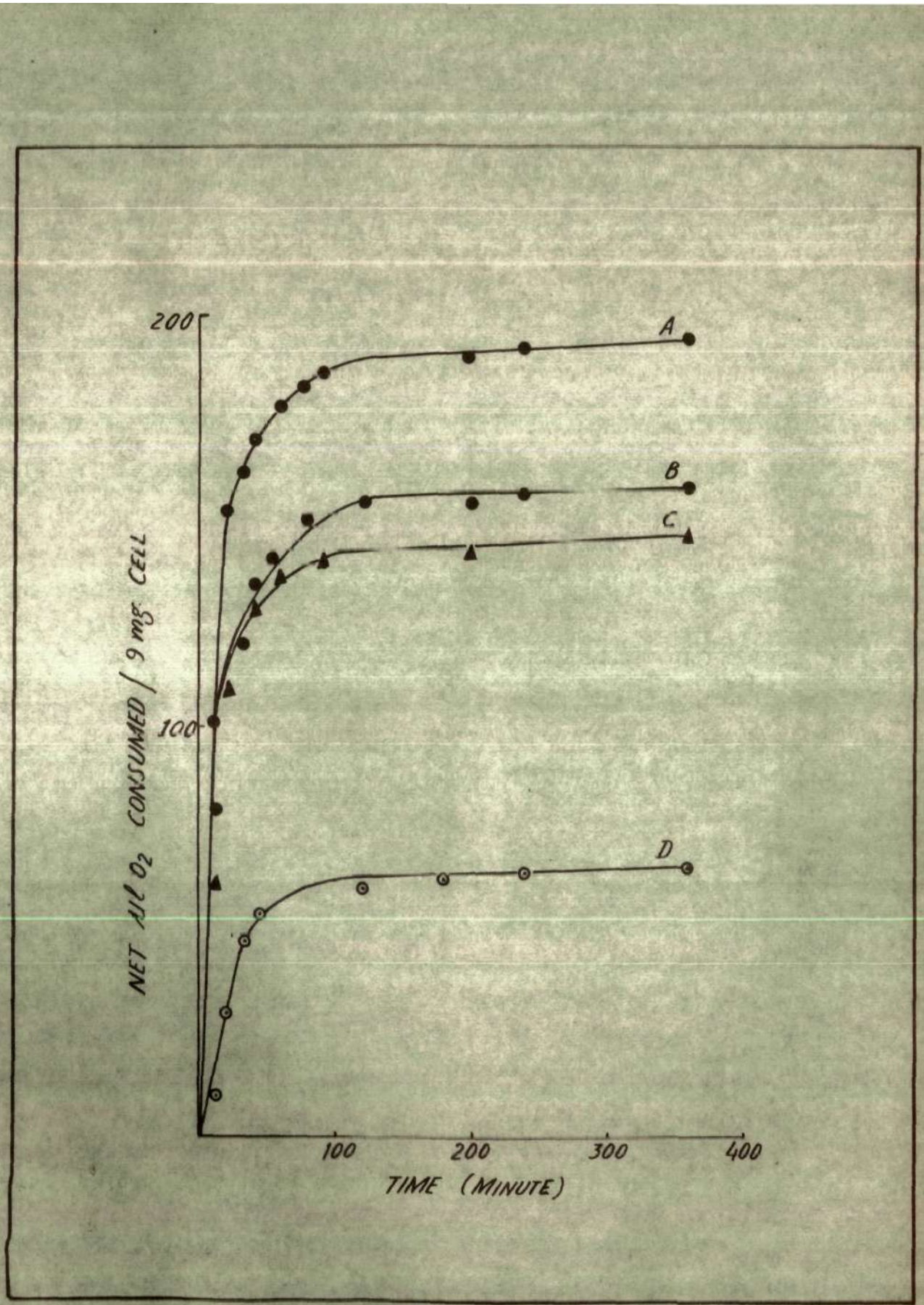

200
100
A
B
C
D
NET µl O2 CONSUMED / 9 mg CELL
100
200
300
400
TIME (MINUTE)

Fig.6. Ability of benzaldehyde-grown cells to oxidize benzaldehyde, succinate, 2,4-dihydroxy benzaldehyde and salicylaldehyde. Each Warburg flask contained 9 mg dry weight equivalent of benzaldehyde-grown cells and 2 μ mole substrate.

A ... Benzaldehyde

B ... Succinate

C ... 2,4-dihydroxy benzaldehyde

D ... Salicylaldehyde

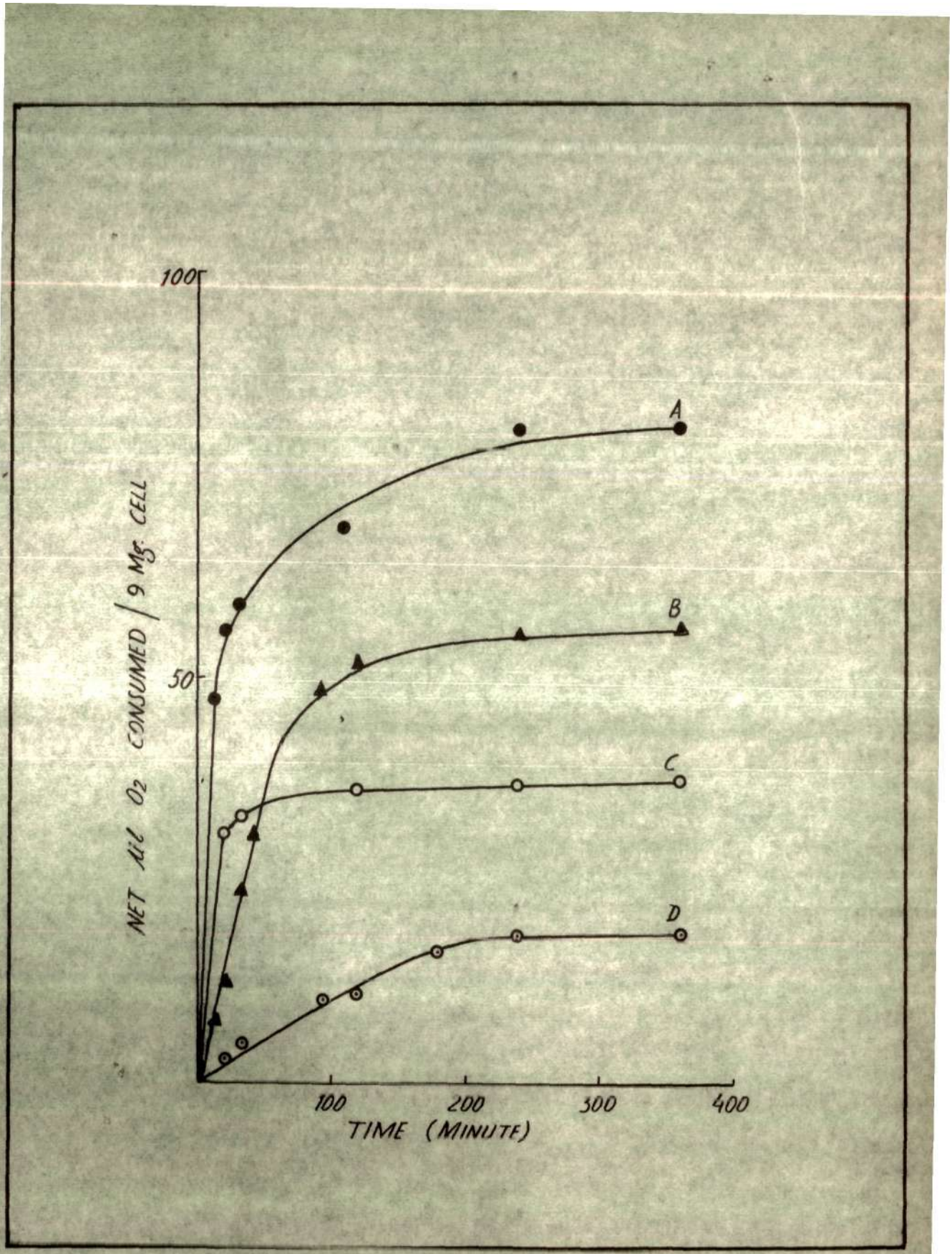

100
50
NET µl O2 CONSUMED / 9 Mg. CELL
A
B
C
D
100
200
300
400
TIME (MINUTE)

Fig.7. Ability of benzaldehyde-grown cells to oxidize adipate, p-hydroxy benzoate and m-hydroxy benzoate. Each Warburg flask contained 9 mg dry weight equivalent of benzaldehyde-grown cells and 2 μ mole substrate.

A ... Adipate

B ... p-hydroxy benzoate

C ... m-hydroxy benzoate

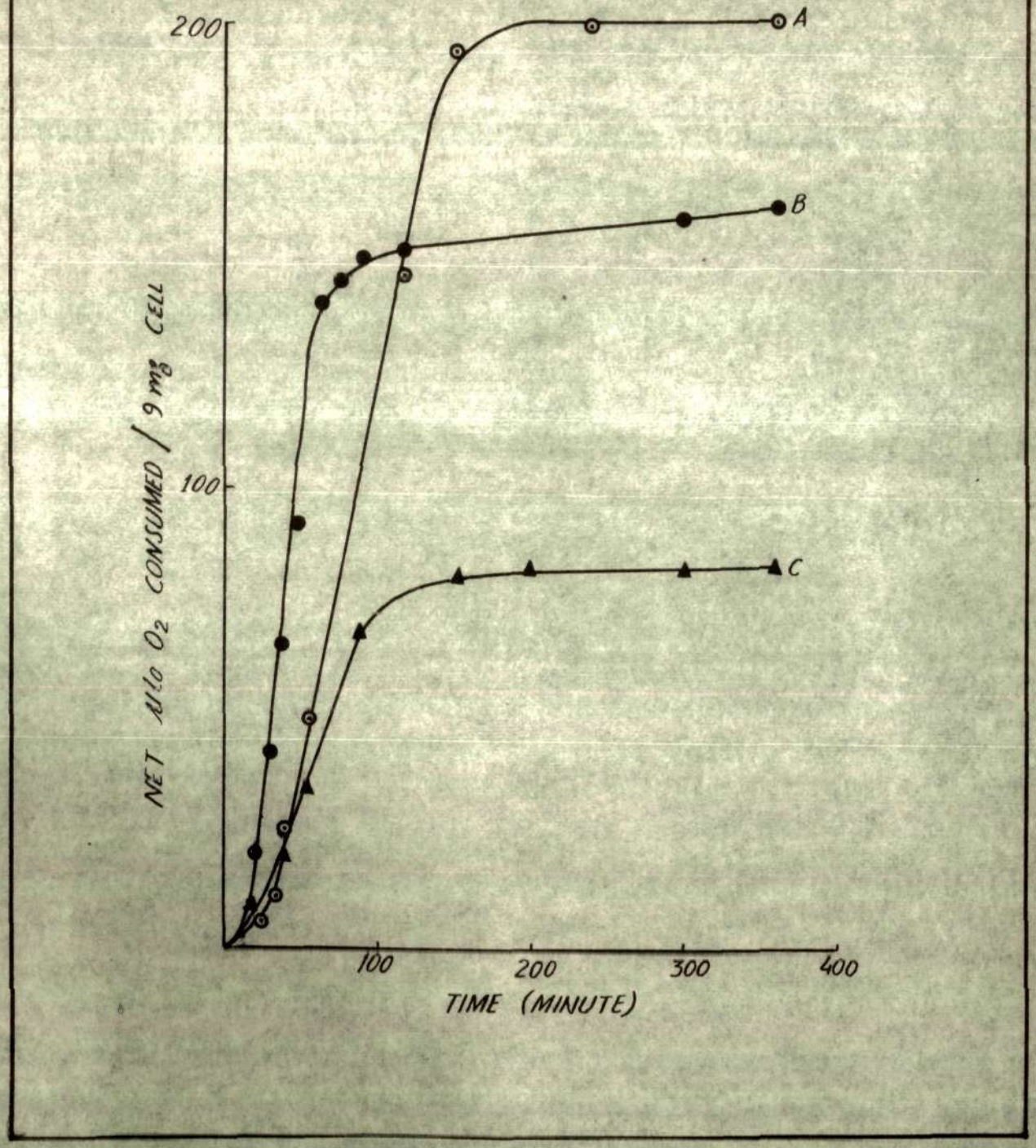

200
100
A
B
C
NET µl. O_2 CONSUMED / 9 mg CELL
100
200
300
400
TIME (MINUTE)

Fig.8. Ability of benzaldehyde-grown cells to oxidise α-ketoglutarate, sodium citrate, fumarate and malate. Each Warburg flask contained 4 mg dry weight equivalent of benzaldehyde-grown cells and 2 µ mole substrate.

A ... α-ketoglutarate

B ... Sodium citrate

C ... Fumarate

D ... Malate

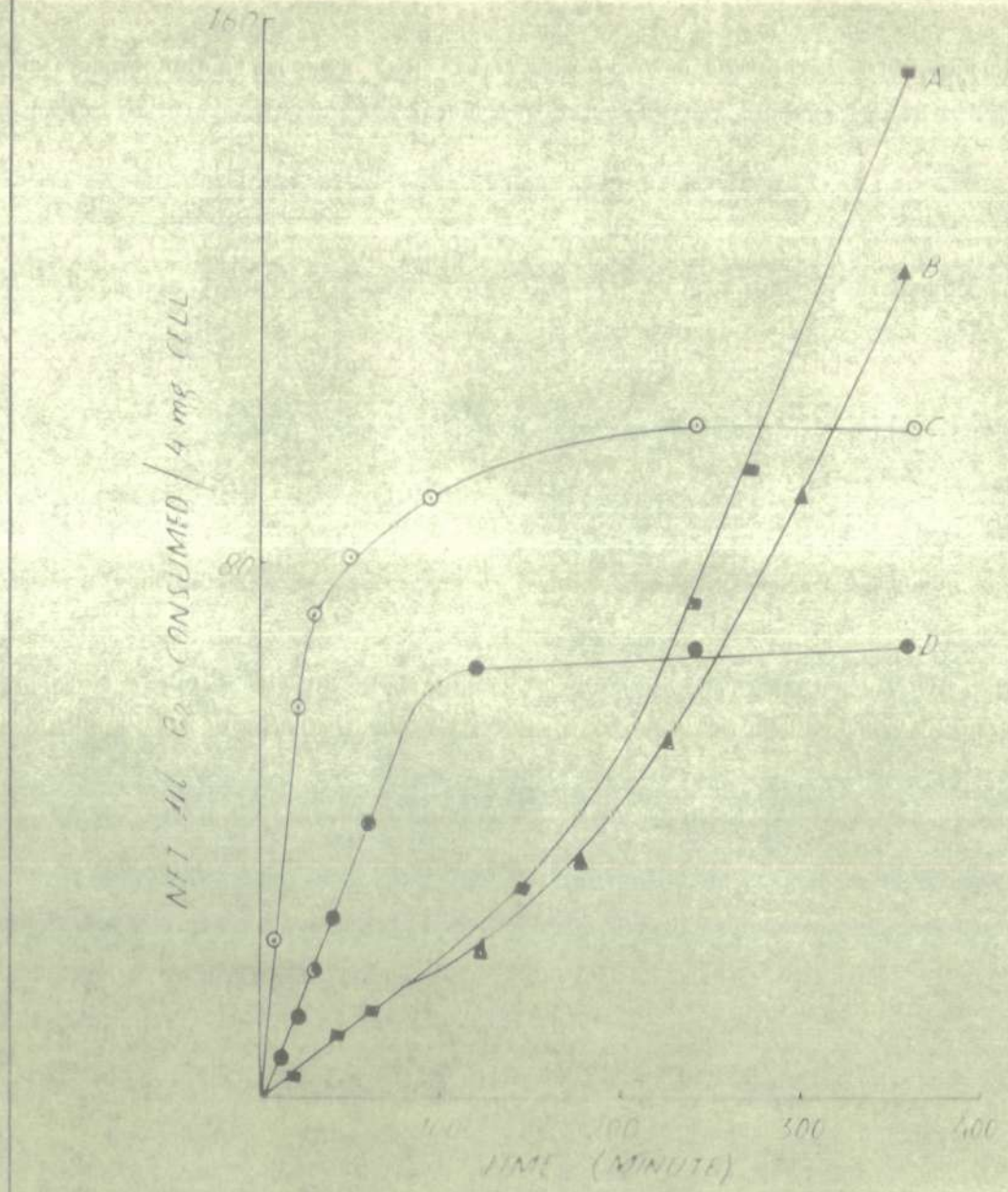

160
80
A
B
C
D
NET ML O2 CONSUMED / 4 mg CELL
300
400
TIME (MINUTE)

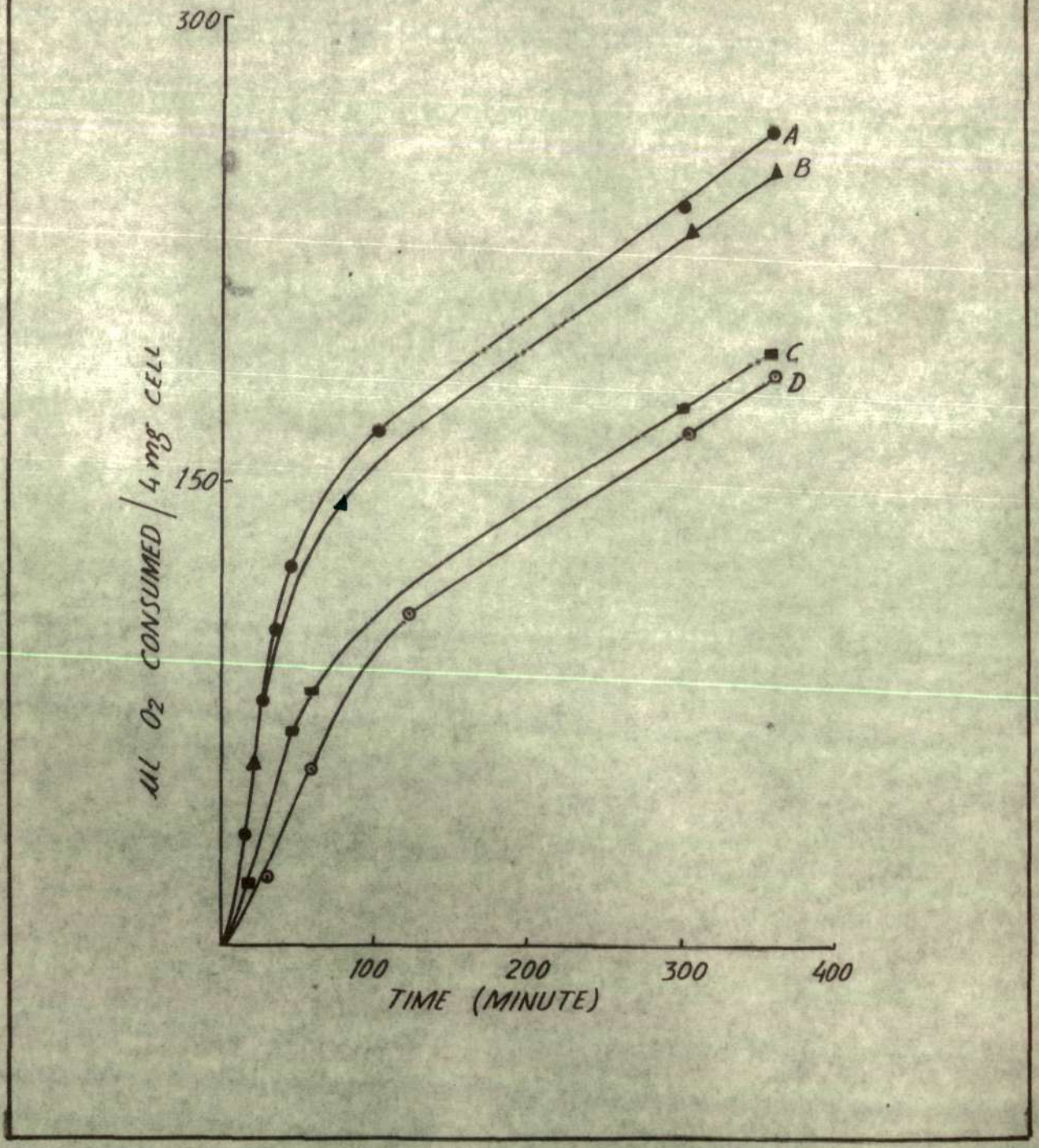
Fig.9. Ability of benzaldehyde-grown cells to oxidize benzaldehyde in presence of puromycin. Each Warburg flask contained 4 mg dry weight equivalent of benzaldehyde-grown cells, 0.6 μ mole puromycin and 2 μ mole benzaldehyde. The endogenous flask niether contained substrate nor puromycin.

A ... Benzaldehyde

B ... Benzaldehyde + Puromycin

C ... Puromycin

D ... Endogenous


300
150
µl O2 CONSUMED / 4 mg CELL
A
B
C
D
100
200
300
400
TIME (MINUTE)

and total oxygen uptake was considerably decreased in presence of these protein synthesis inhibitors (Figs. 10,11,13,14). Chloramphenicol (0.5 mM) did not have any effect on the rate of oxidation of benzaldehyde (Fig.12) and on the duration of lag phase in the oxidation of p-hydroxy benzoate and m-hydroxy benzoate (Figs. 13,14) whereas puromycin markedly prolonged the lag phase (Figs. 10,11). o-nitrobenzoic acid is known to interfere with protein synthesis in bacterial system (Montgomery and Durham , 1970). As compared to puromycin 10 mM o-nitroben - zoic acid markedly decreased the total oxygen uptake of benzaldehyde but had no effect on the rate of its oxidation (Fig.15). Although it affected the rate of oxidation of p-hydroxy benzoate (Fig.16) and m-hydroxy benzoate (Fig.17) , it had no significant effect on the total oxygen uptake. In contrast to puromycin and chloramphenicol the duration of lag period in the oxidation of p-hydroxy benzoate and m-hydroxy benzoate was not significantly affected.

(ii) Benzoate-grown cells - The rate of the oxidation of the same compounds were tested by the cells grown on benzoate. The pattern of oxygen

Fig.10. Ability of benzaldehyde-grown cells to oxidise p-hydroxy benzoate in presence of puromycin. Each Warburg flask contained 4 mg dry weight equivalent of benzaldehyde-grown cells, 0.6 μ mole puromycin and 2 μ mole p-hydroxy benzoate. The endogenous flask neither contained substrate nor puromycin.

A ... p-hydroxy benzoate + puromycin

B ... p-hydroxy benzoate

C ... Puromycin

D ... Endogenous

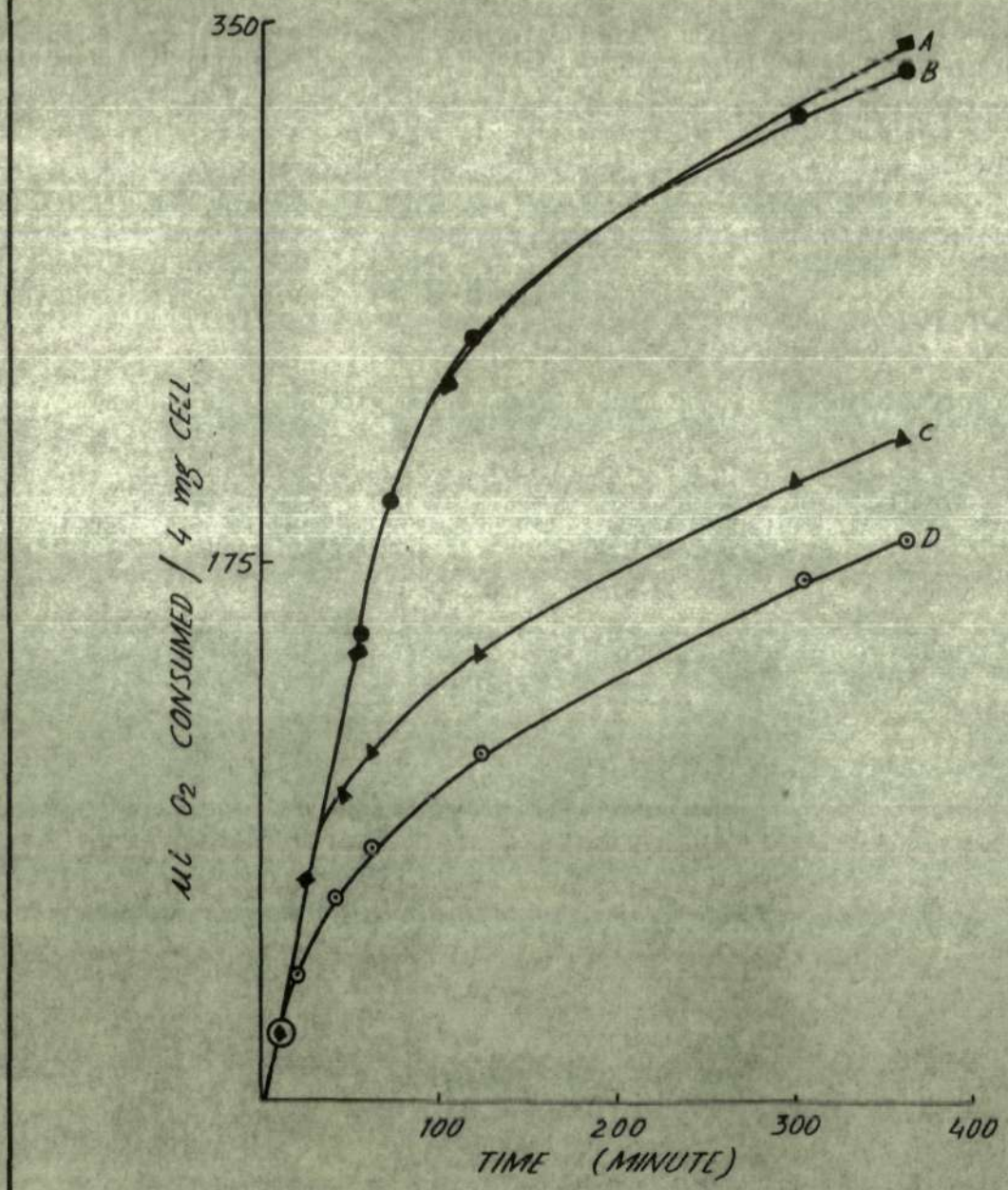

350
175
µl O2 CONSUMED / 4 mg CELL
A
B
C
D
100
200
300
400
TIME (MINUTE)

Fig.11. Ability of benzaldehyde-grown cells to oxidize m-hydroxy benzoate in presence of puromycin. Each Warburg flask contained 4 mg dry weight equivalent of benzaldehyde-grown cells, 0.6 μ mole puromycin and 2 μ mole m-hydroxy benzoate. The endogenous flask niether contained substrate nor puromycin.

A ... m-hydroxy benzoate

B ... m-hydroxy benzoate + Puromycin

C ... Puromycin

D ... Endogenous

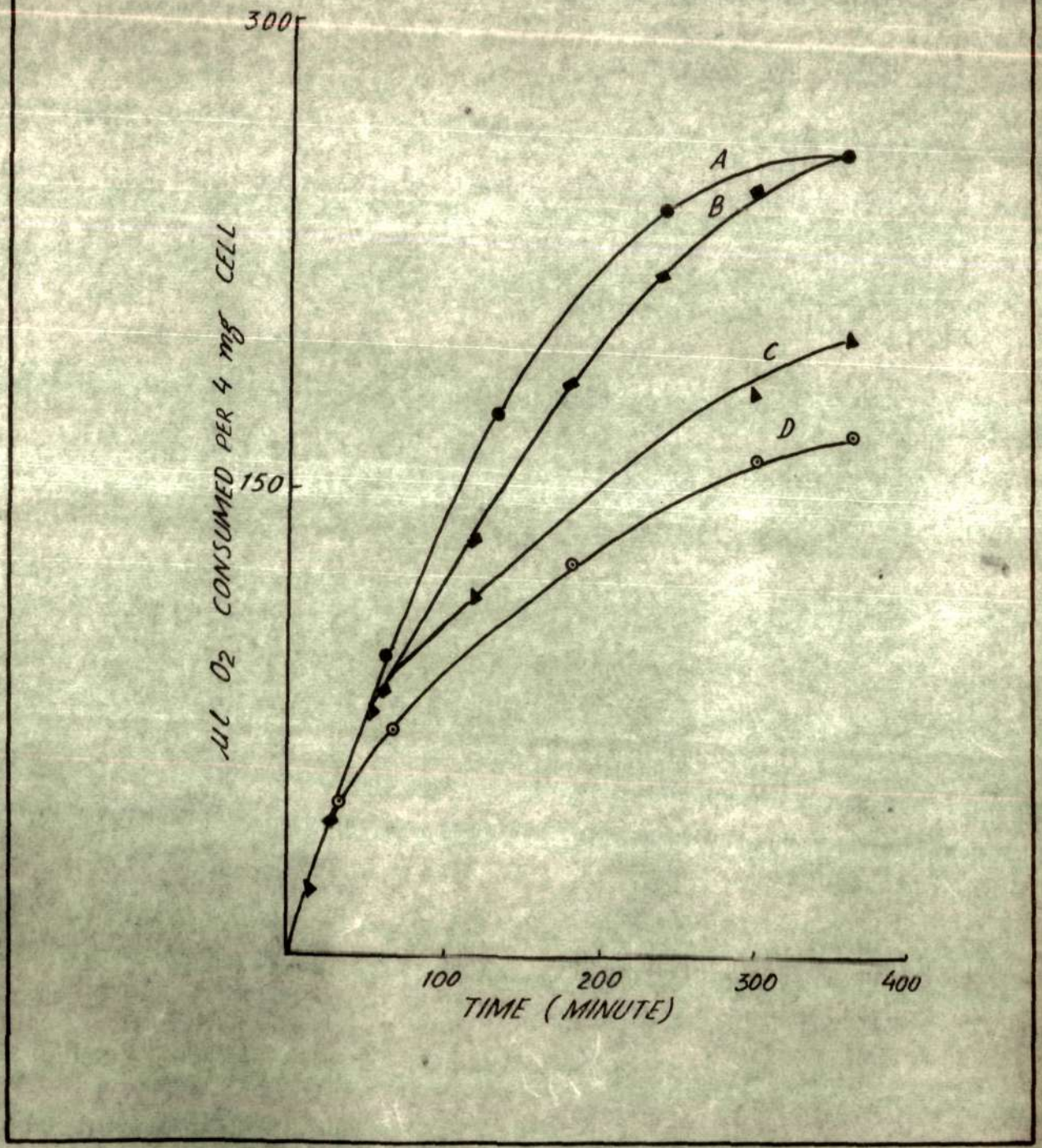

300
150
µl O2 CONSUMED PER 4 mg CELL
A
B
C
D
100
200
300
400
TIME (MINUTE)

Fig.12. Ability of benzaldehyde-grown cells to oxidize benzaldehyde in presence of chloramphenicol. Each Warburg flask contained 4 mg dry weight equivalent of benzaldehyde-grown cells, 1 μ mole chloramphenicol and 2 μ mole benzaldehyde. The endogenous flask niether contained substrate nor chloramphenicol.

A ... Benzaldehyde

B ... Benzaldehyde + Chloramphenicol

C ... Endogenous

D ... Chloramphenicol

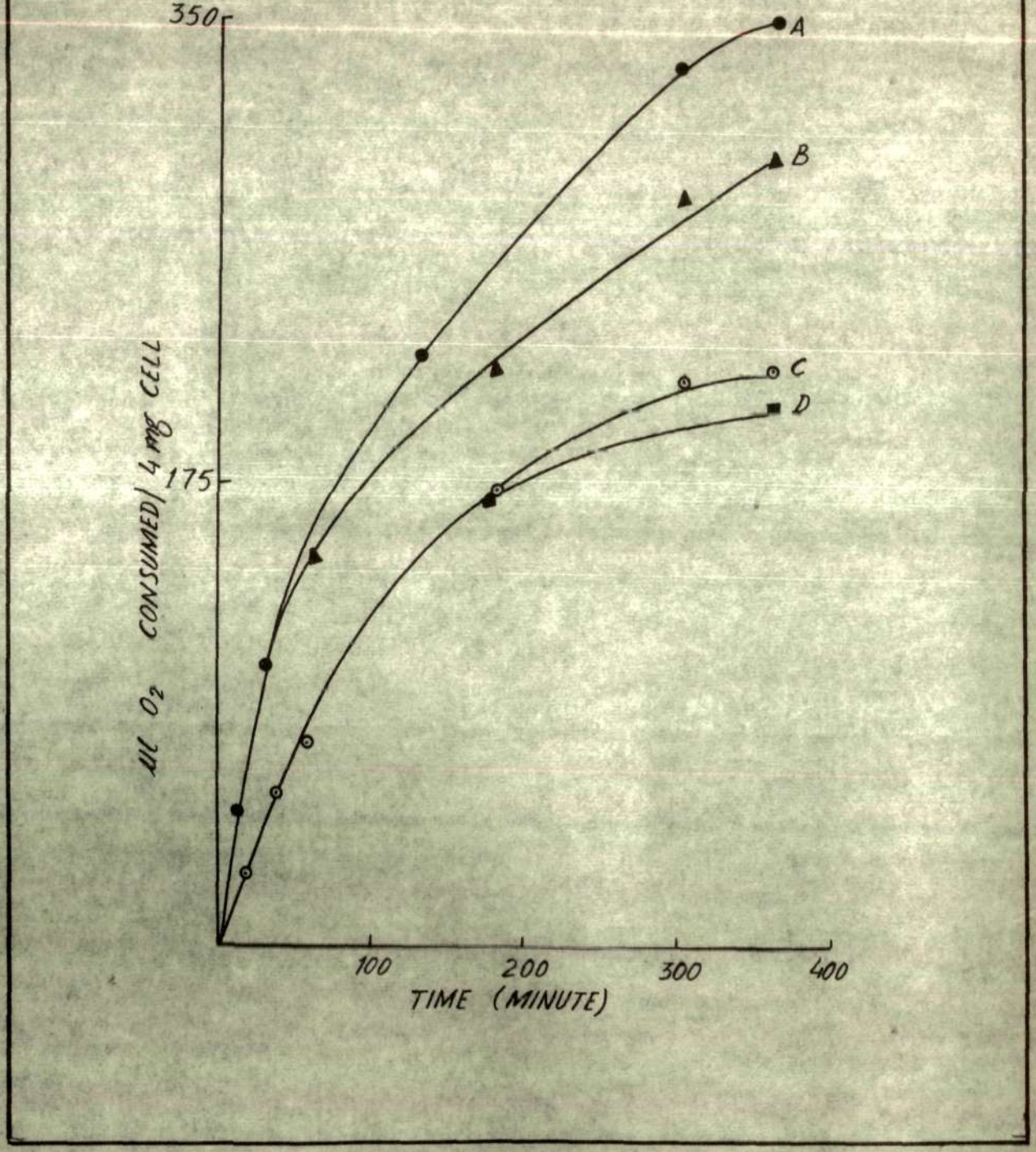

350
175
µl O2 CONSUMED/ 4 mg CELL
A
B
C
D
100
200
300
400
TIME (MINUTE)

Fig.13. Ability of benzaldehyde-grown cells to oxidize p-hydroxy benzoate in presence of chloramphenicol. Each Warburg flask contained 4 mg dry weight equivalent of benzaldehyde-grown cells, 1 μ mole chloramphenicol and 2 μ mole p-hydroxy benzoate. The endogenous flask niether contained substrate nor chloramphenicol.

A ... p-hydroxy benzoate + Chloramphenicol

B ... p-hydroxy benzoate

C ... Endogenous

D ... Chloramphenicol

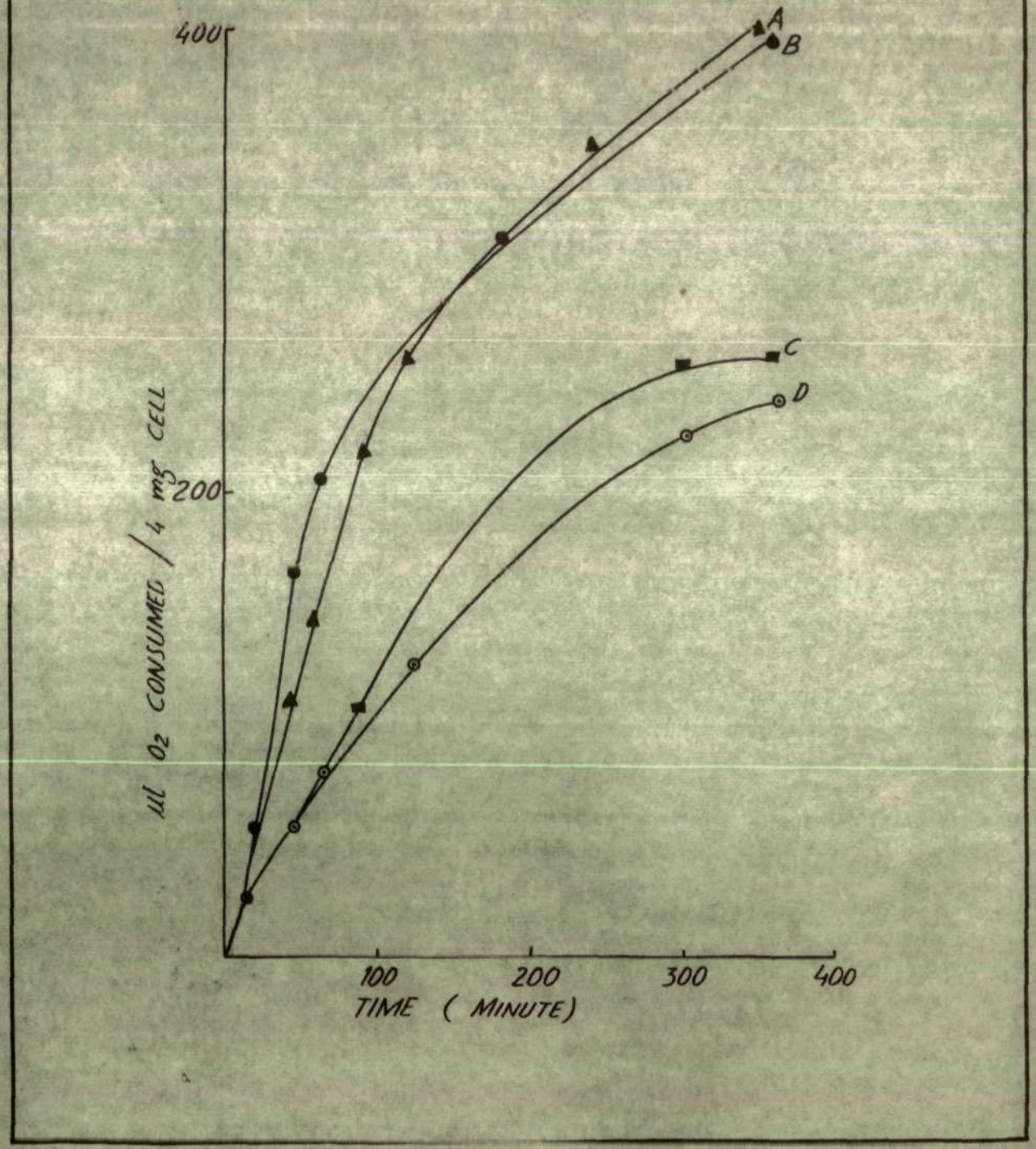

400
200
A
B
C
D
µl O2 CONSUMED / 4 mg CELL
100
200
300
400
TIME (MINUTE)

Fig.14. Ability of benzaldehyde-grown cells to oxidize m-hydroxy benzoate in presence of chloramphenicol. Each Warburg flask contained 4 mg dry weight equivalent of benzaldehyde-grown cells, 1 µ mole chloramphenicol and 2 µ mole m-hydroxy benzoate. The endogenous flask niether contained substrate nor chloramphenicol.

A ... m-hydroxy benzoate

B ... m-hydroxy benzoate + Chloramphenicol

C ... Endogenous

D ... Chloramphenicol

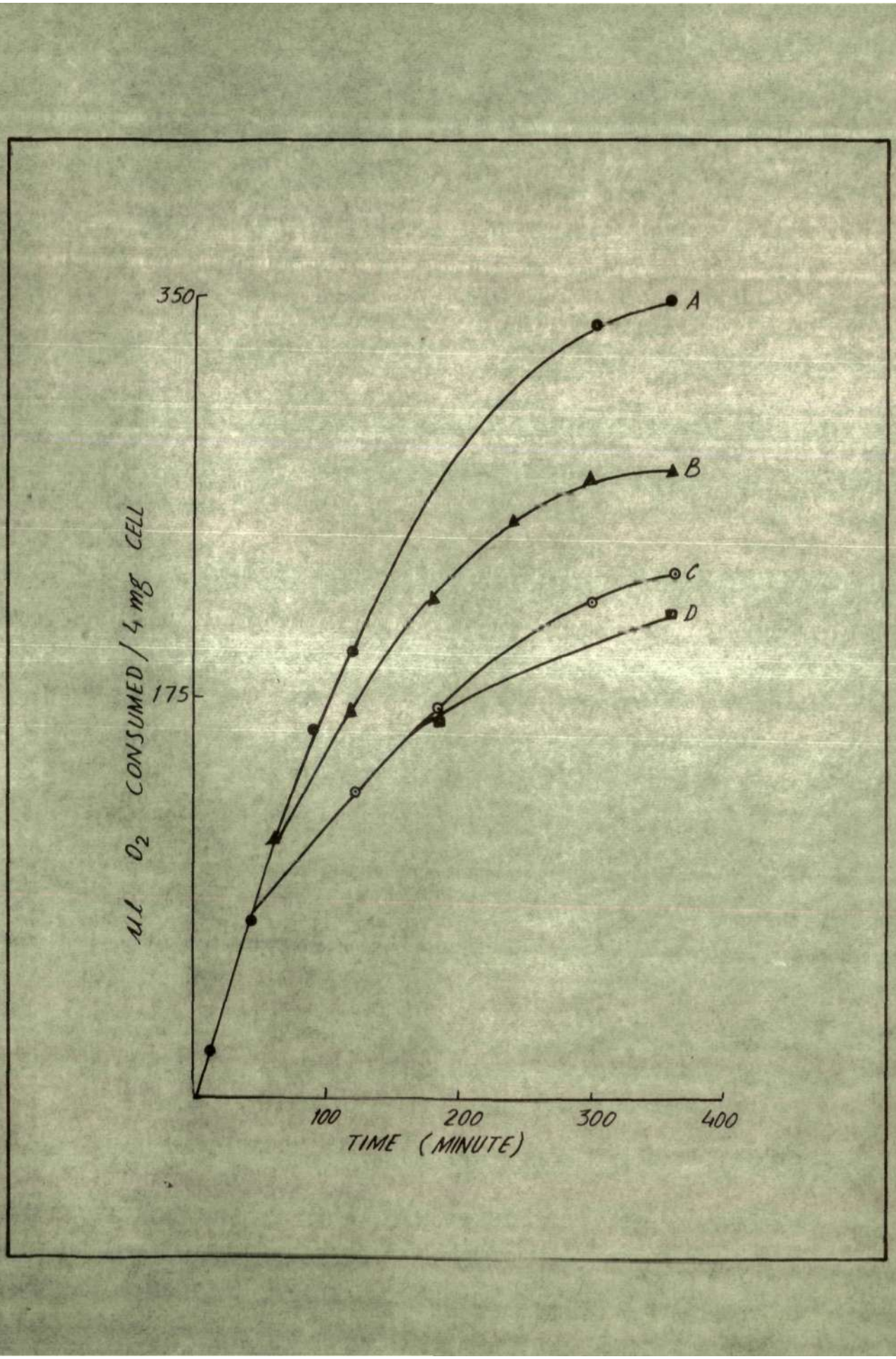

350
175
µl O_2 CONSUMED / 4 mg CELL
A
B
C
D
100
200
300
400
TIME (MINUTE)

Fig.15. Ability of benzaldehyde-grown cells to oxidize benzaldehyde in presence of o-nitrobenzoic acid. Each Warburg flask contained 4 mg dry weight equivalent of benzaldehyde-grown cells, 20 μ mole o-nitrobenzoic acid and 2 μ mole benzaldehyde. The endogenous flask niether contained substrate nor o-nitrobenzoic acid.

A ... Benzaldehyde

B ... Benzaldehyde + o-nitrobenzoic

C ... o-nitrobenzoic acid

D ... Endogenous

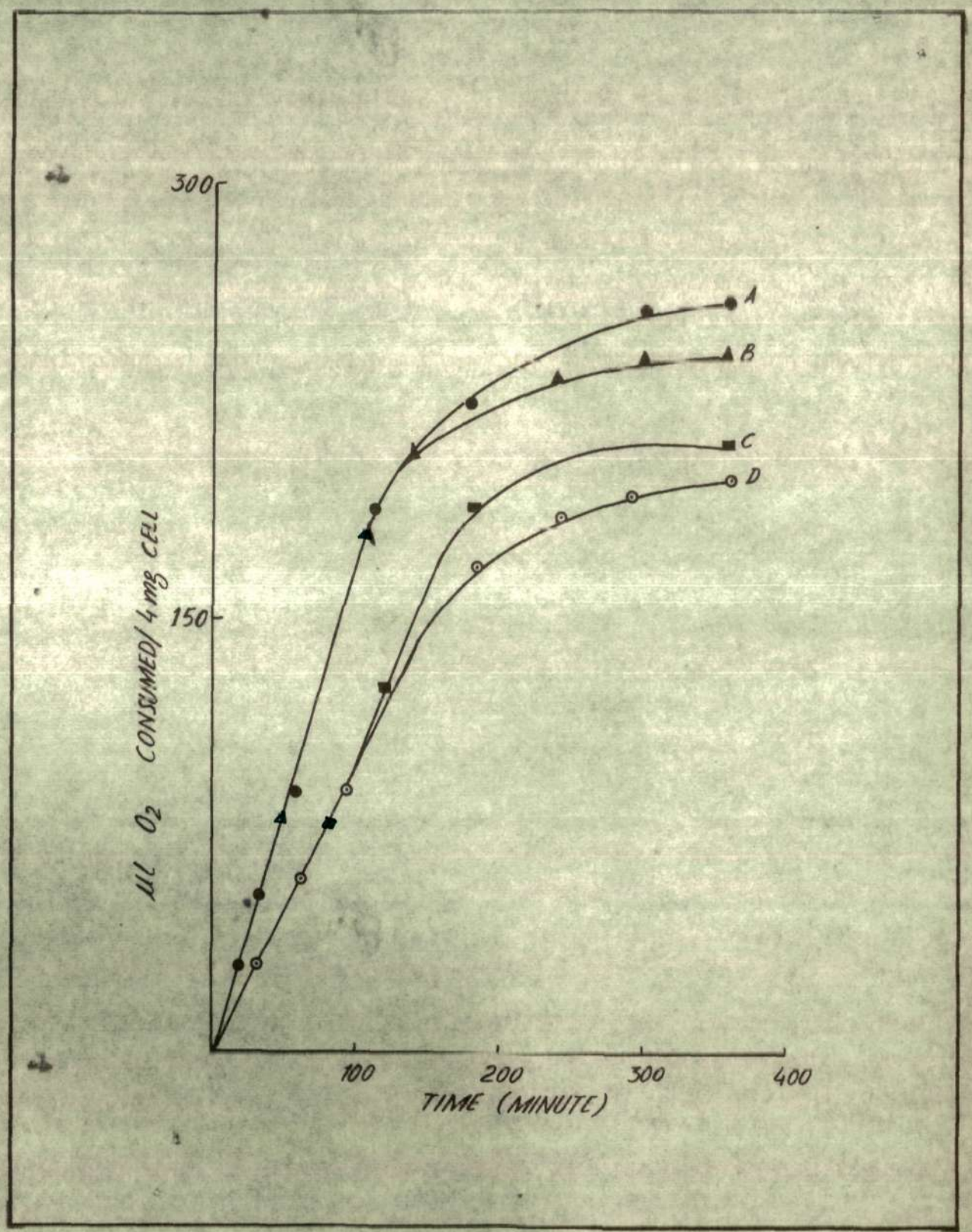

300
150
µl O2 CONSUMED/ 4 mg CEL
A
B
C
D
100
200
300
400
TIME (MINUTE)

Fig.16. Ability of benzaldehyde-grown cells to oxidise p-hydroxy benzoate in presence of o-nitrobenzoic acid. Each Warburg flask contained 4 mg dry weight equivalent of benzaldehyde-grown cells, 20 μ mole o-nitrobenzoic acid and 2 μ mole p-hydroxy benzoate. The endogenous flask niether contained substrate nor o-nitrobenzoic acid .

A ... p-hydroxy benzoate + o-nitrobenzoic acid

B ... p-hydroxy benzoate

C ... o-nitrobenzoic acid

D ... Endogenous

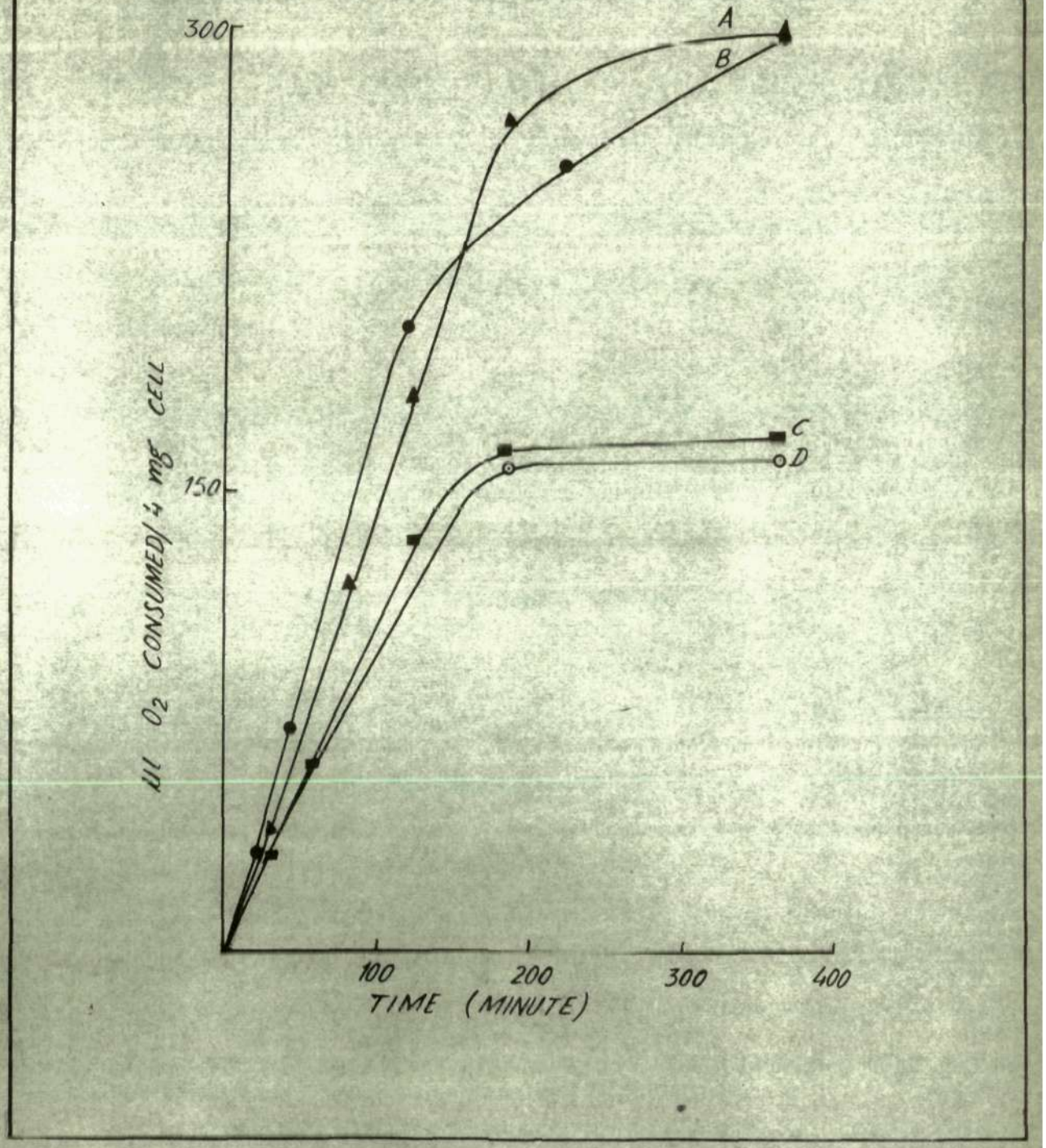

300
150
A
B
C
D
µl O2 CONSUMED/½ mg CELL
100
200
300
400
TIME (MINUTE)

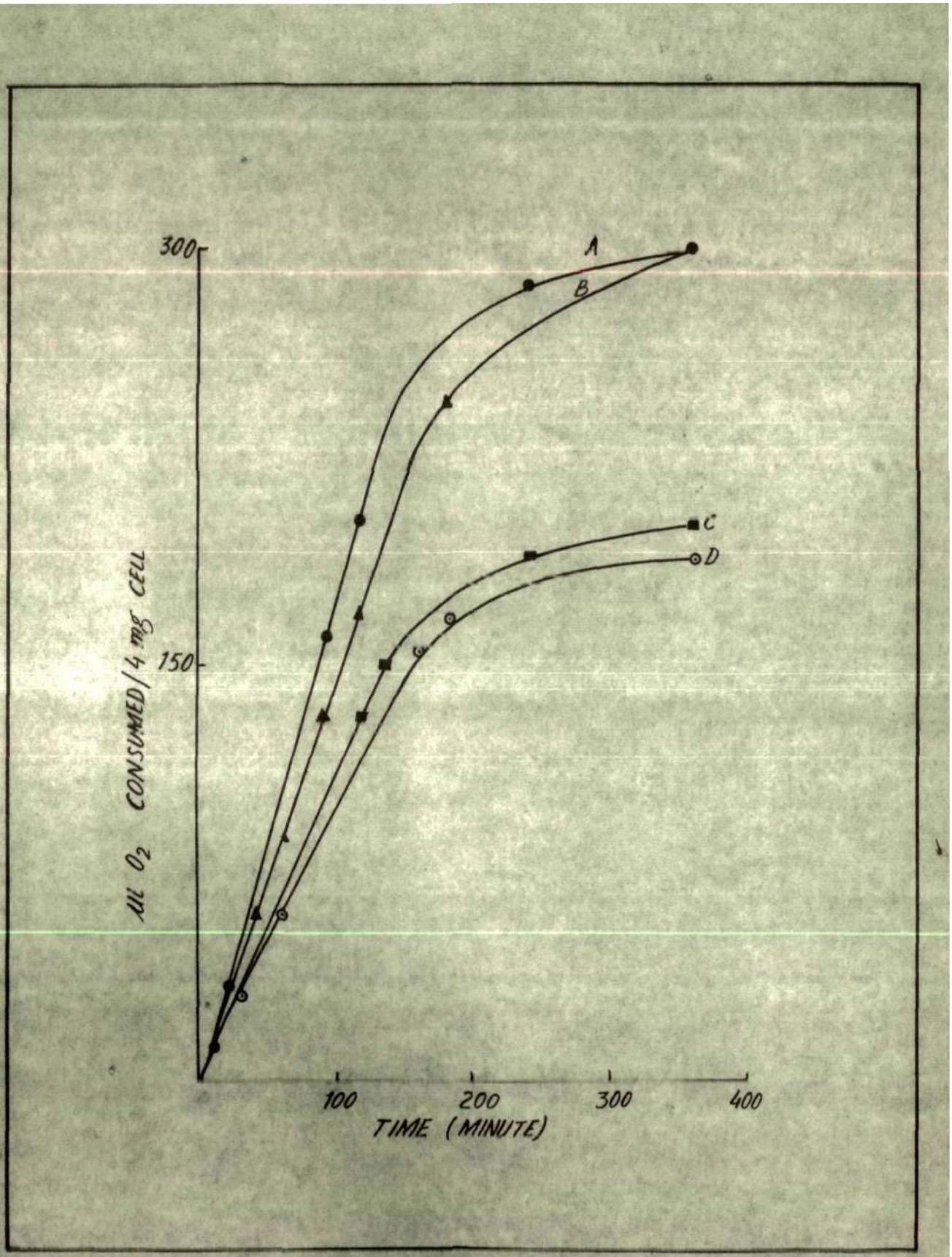
Fig.17. Ability of benzaldehyde-grown cells to oxidize m-hydroxy benzoate in presence of o-nitrobenzoic acid. Each Warburg flask contained 4 mg dry weight equivalent of benzaldehyde-grown cells, 20 µ mole o-nitrobenzoic acid and 2 µ mole m-hydroxy benzoate. The endogenous flask niether contained substrate nor o-nitrobenzoic acid.

A ... m-hydroxy benzoate

B ... m-hydroxy benzoate + o-nitrobenzoic acid

C ... o-nitrobenzoic acid

D ... Endogenous


300
150
A
B
C
D
100
200
300
400
TIME (MINUTE)
µl O2 CONSUMED/4 mg CELL

uptake is shown in Figs. 18, 19 and 20. Although benzaldehyde was oxidised without lag, the total oxygen uptake was reduced to about 50% of that utilized by benzaldehyde-grown cells. Protocatechuate, catechol, 2,4-dihydroxy benzaldehyde, succinate and acetate were oxidised without lag. These cells did not oxidise salicylaldehyde. Like benzaldehyde-grown cells, these cells also oxidised p-hydroxy benzoate, m-hydroxy benzoate and adipate with lag phase.

(iii) p-Hydroxy benzoate-grown cells - These cells oxidised benzaldehyde, benzoate and m-hydroxy benzoate with lag (Fig.21) whereas p-hydroxy benzoate, adipate, 2,4-dihydroxy benzaldehyde, protocatechuate, catechol, succinate and acetate were oxidised without lag (Figs. 22,23). The oxygen uptake of 2,4-dihydroxy benzaldehyde continued to increase slowly even upto 6 hours (Fig.23).

(iv) m-Hydroxy benzoate-grown cells - These cells oxidised benzaldehyde, benzoate, 2,4-dihydroxy benzaldehyde, p-hydroxy benzoate and adipate with lag. Protocatechuate, m-hydroxy benzoate, catechol, succinate and acetate were oxidised without lag. The

Fig.18. Ability of benzoate-grown cells to oxidize succinate, acetate, benzaldehyde and 2,4-dihydroxy benzaldehyde. Each Warburg flask contained 8 mg dry weight equivalent of benzoate-grown cells and 2 μ mole substrate.

A ... Succinate

B ... Acetate

C ... Benzaldehyde

D ... 2,4-dihydroxy benzaldehyde

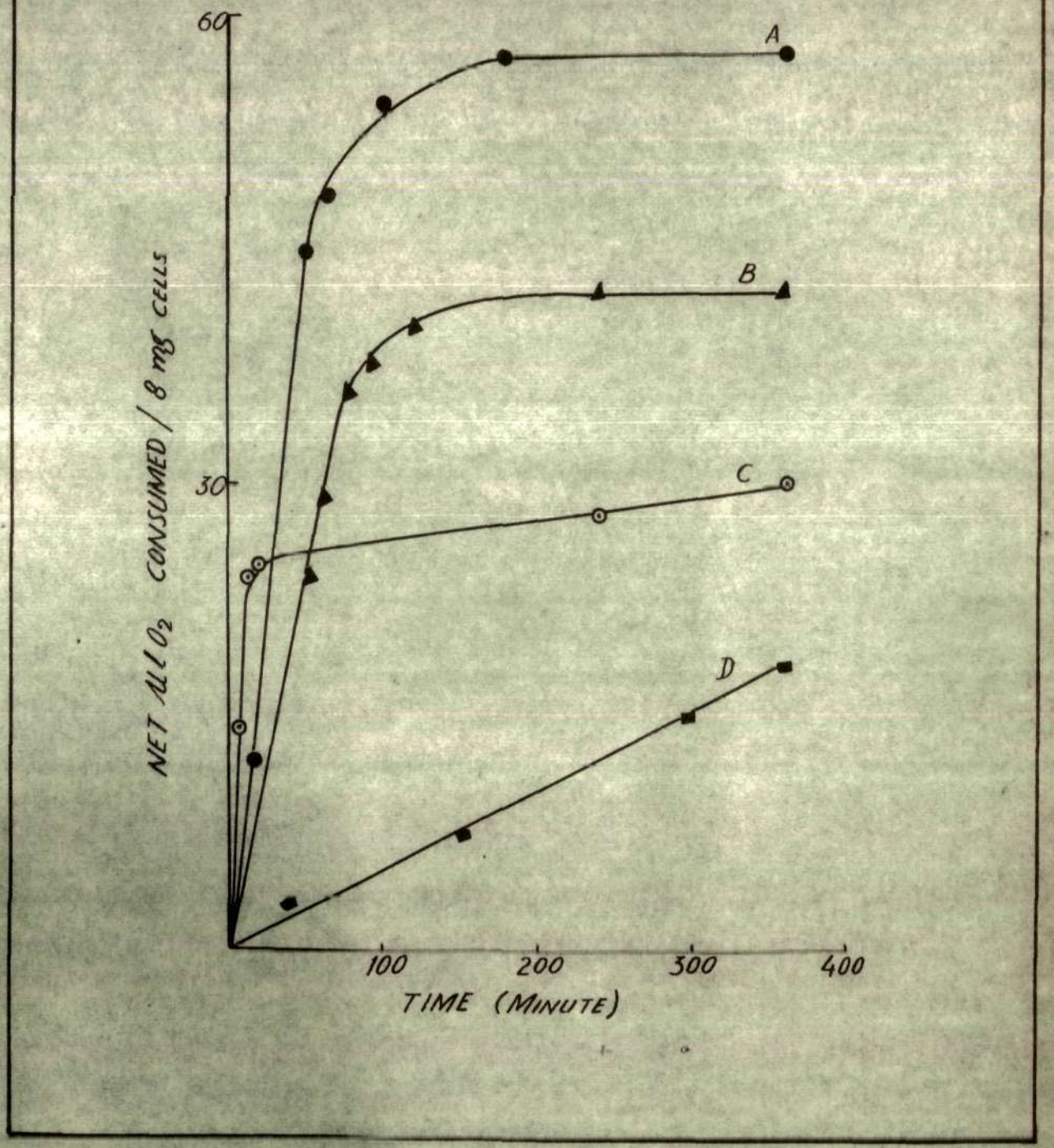

60
30
A
B
C
D
NET µl O_2 CONSUMED / 8 mg CELLS
100
200
300
400
TIME (MINUTE)

Fig.19. Ability of benzoate-grown cells to oxidise benzoate, protocatechuate and catechol. Each Warburg flask contained 8 mg dry weight equivalent of benzoate-grown cells and 2 μ mole substrate.

A ... Benzoate

B ... Protocatechuate

C ... Catechol

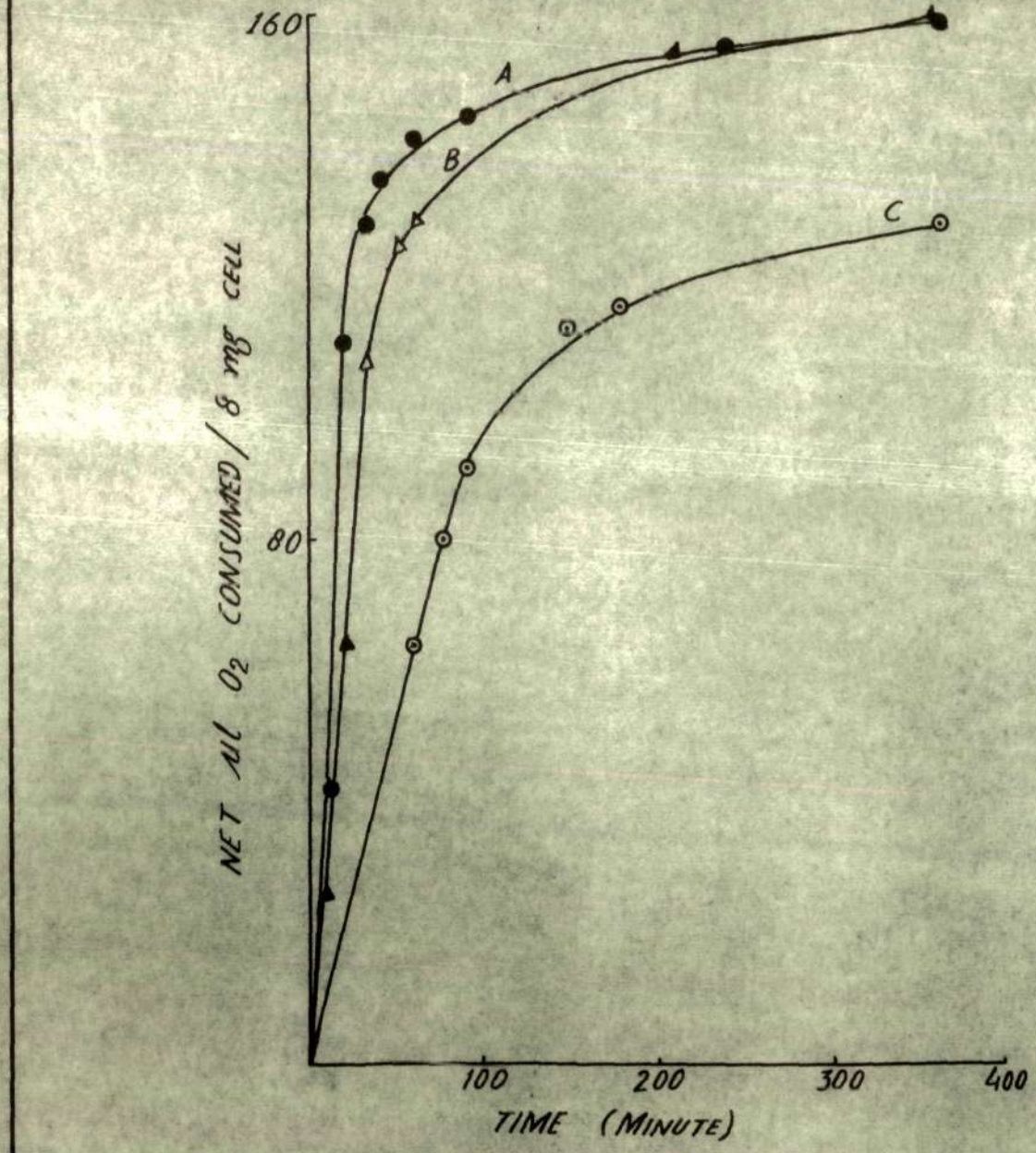

160
80
A
B
C
NET ml O_2 CONSUMED / 8 mg CELL
100
200
300
400
TIME (MINUTE)

Fig.20. Ability of benzoate-grown cells to oxidize p-hydroxy benzoate, adipate and m-hydroxy benzoate. Each Warburg flask contained 8 mg dry weight equivalent of benzoate-grown cells and 2 µ mole substrate.

A ... p-hydroxy benzoate

B ... Adipate

C ... m-hydroxy benzoate

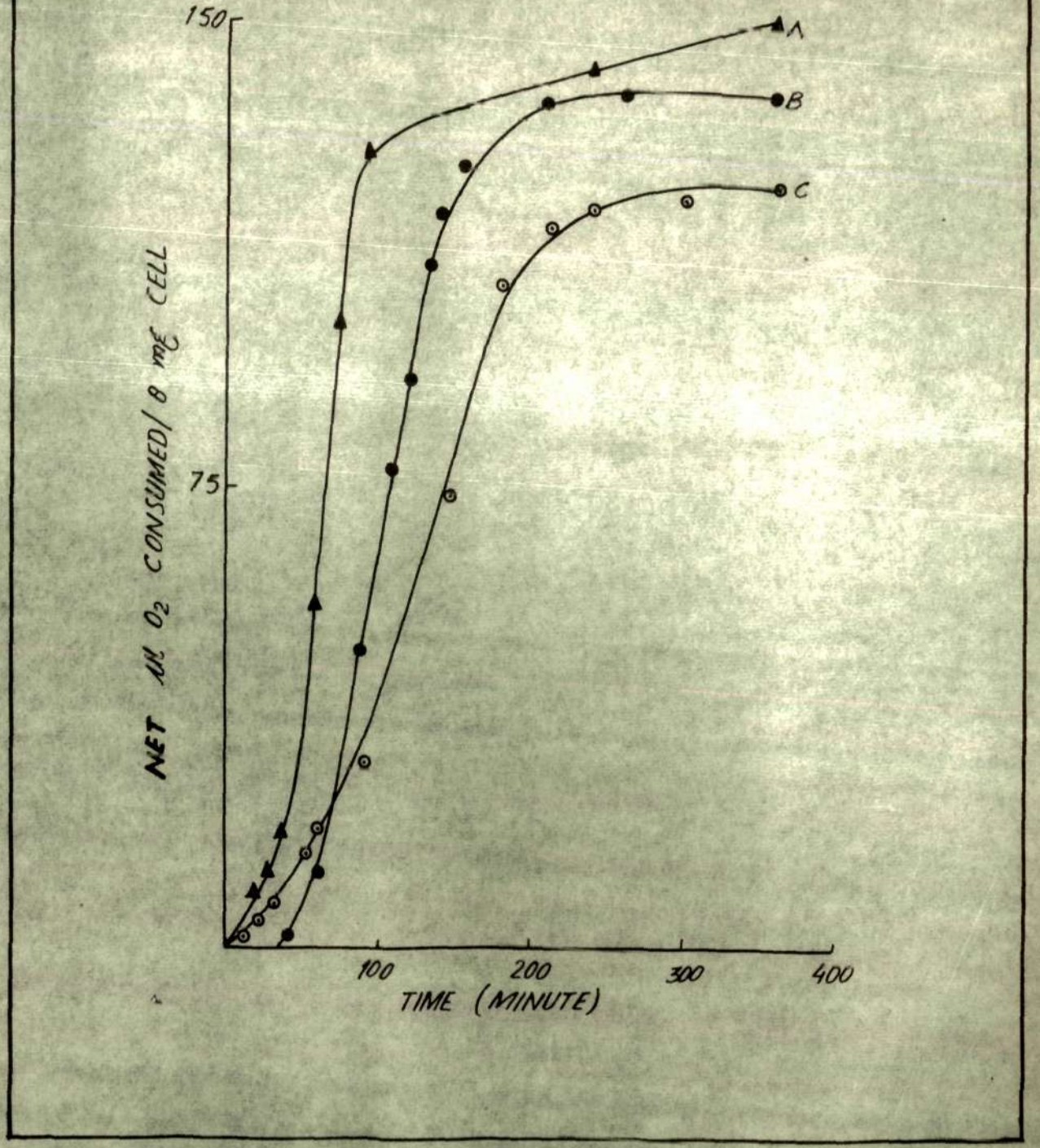

150
75
NET µl O2 CONSUMED/ 8 mg CELL
A
B
C
100
200
300
400
TIME (MINUTE)

Fig.21. Ability of p-hydroxy benzoate-grown cells to oxidize benzoate, m-hydroxy benzoate and benzaldehyde. Each Warburg flask contained 13 mg dry weight equivalent of p-hydroxy benzoate-grown cells and 2 μ mole substrate.

A ... Benzoate

B ... m-hydroxy benzoate

C ... Benzaldehyde

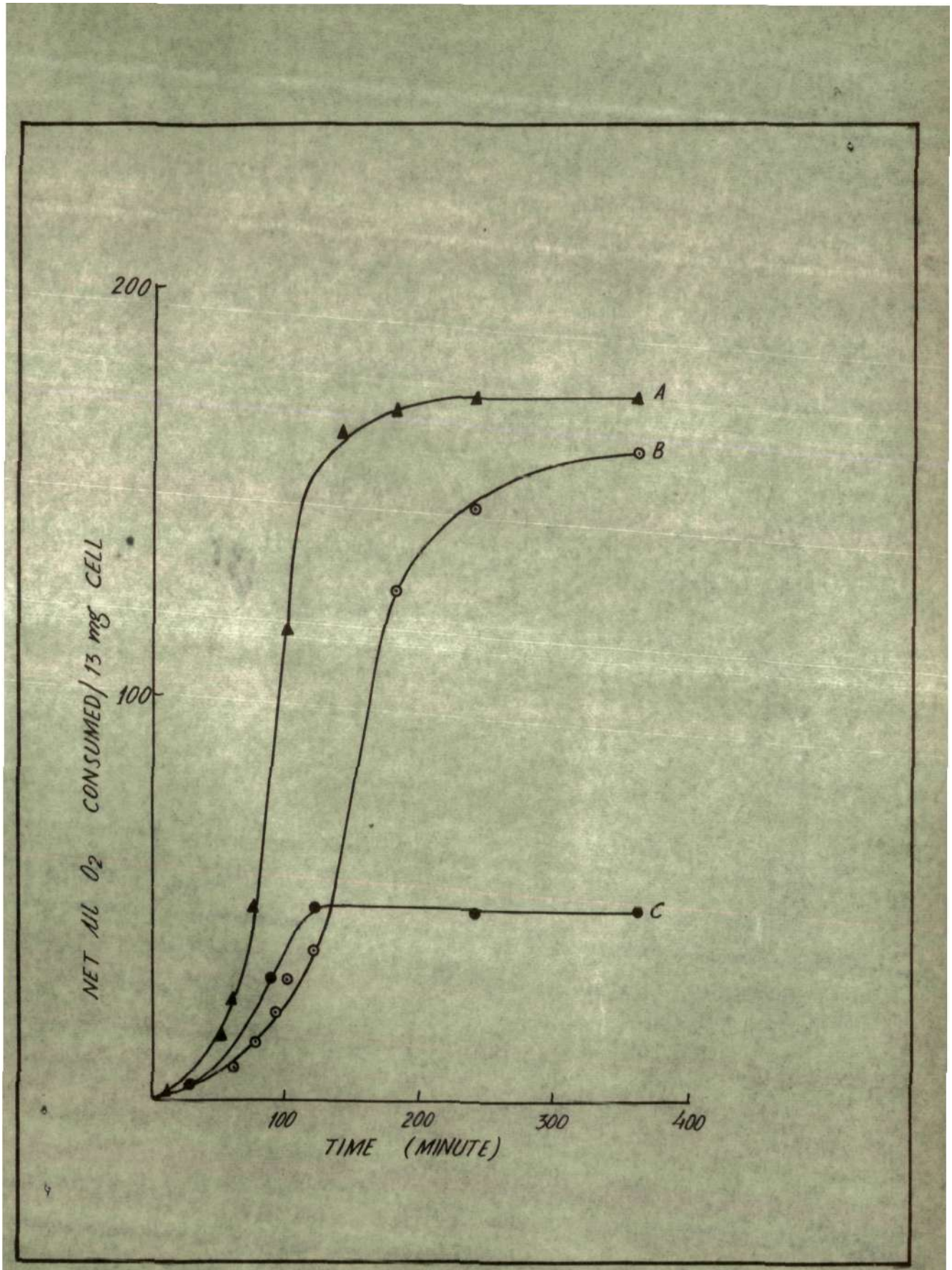

200
100
NET µl O_2 CONSUMED/ 13 mg CELL
A
B
C
100
200
300
400
TIME (MINUTE)

Fig. 22. Ability of p-hydroxy benzoate-grown cells to oxidise p-hydroxy benzoate, adipate, protocatechuate and acetate. Each Warburg flask contained 13 mg dry weight equivalent of p-hydroxy benzoate-grown cells and 2 µ mole substrate.

A ... p-hydroxy benzoate

B ... Adipate

C ... Protocatechuate

D.... Acetate

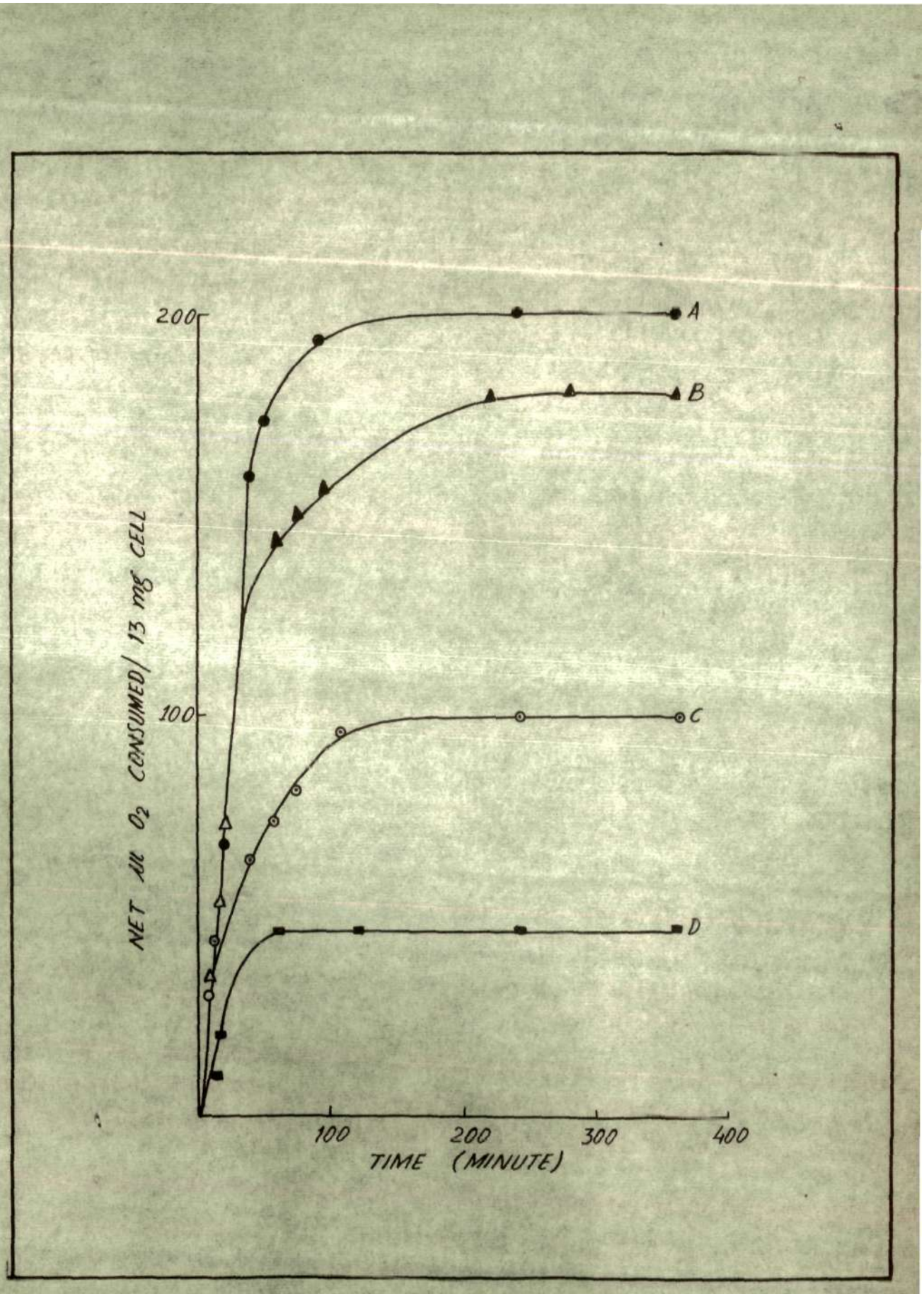
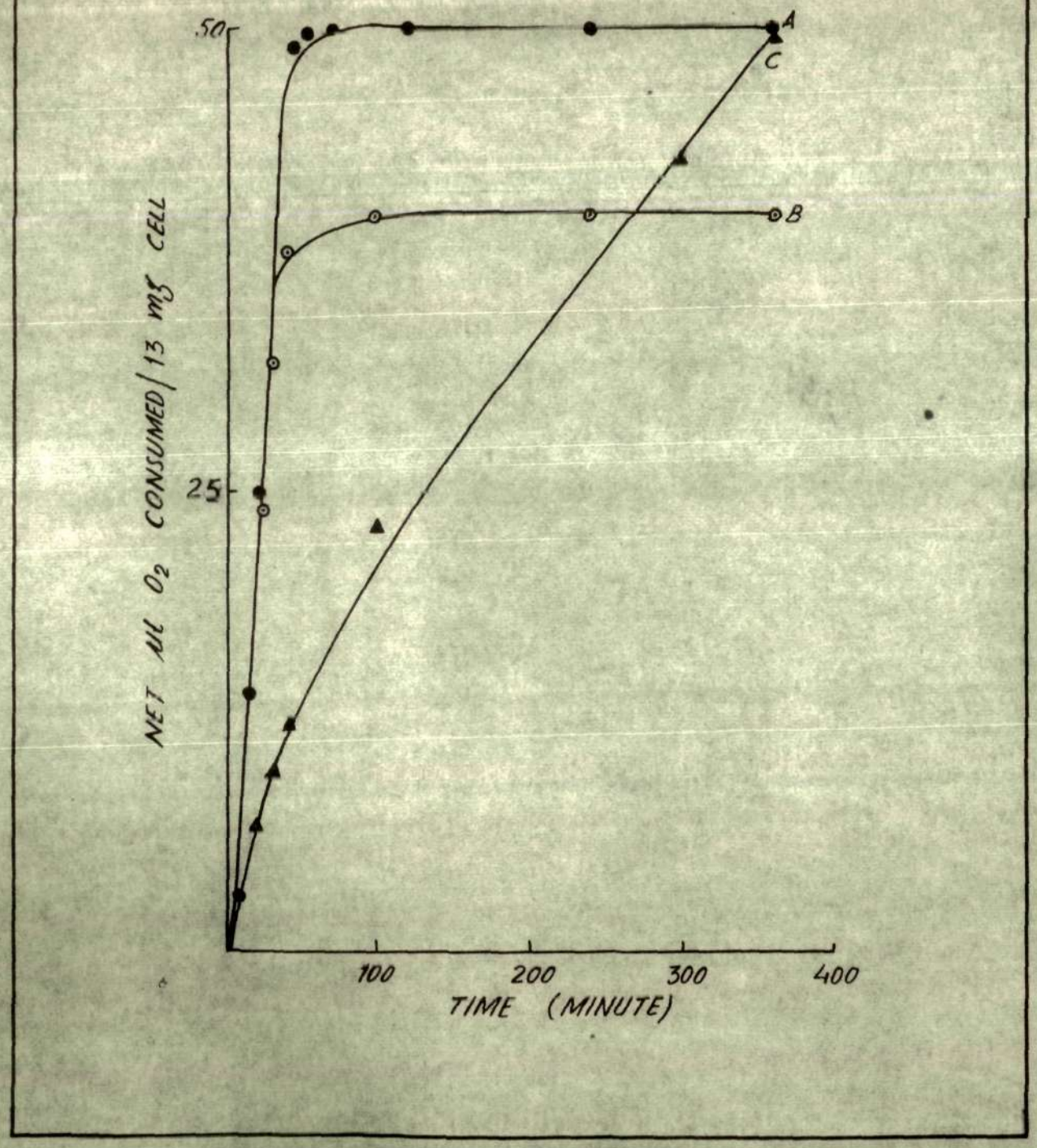

200
100
A
B
C
D
NET μl O_2 CONSUMED/ 13 mg CELL
100
200
300
400
TIME (MINUTE)

Fig.23. Ability of p-hydroxy benzoate-grown cells to oxidize succinate, catechol and 2,4-dihydroxy benzaldehyde. Each Warburg flask contained 13 mg dry weight equivalent of p-hydroxy benzoate-grown cells and 2 μ mole substrate.

A ... Succinate

B ... Catechol

C ... 2,4-dihydroxy benzaldehyde


50
25
A
C
B
NET µl O2 CONSUMED/13 mg CELL
100
200
300
400
TIME (MINUTE)

oxygen uptake was poor with catechol (Figs. 24,25,26).

(v) Adipate-grown cells - Benzaldehyde , 2,4-dihydroxy benzaldehyde, protocatechuate, adipate , succinate, acetate were oxidised without lag but benzoate, p-hydroxy benzoate and m-hydroxy benzoate were oxidised with lag (Figs. 27, 28,29). These cells did not oxidise catechol.

(vi) Acetate-grown cells - These cells oxidised benzaldehyde, benzoate, p-hydroxy benzoate, m-hydroxy benzoate, protocatechuate, catechol and adipate with lag whereas 2,4-dihydroxy benzaldehyde, succinate and acetate oxidation did not show any lag (Figs. 30, 31, 32).

(vii) Succinate-grown cells - These cells oxidised benzaldehyde, benzoate, protocatechuate, 2,4-dihydroxy benzaldehyde, catechol, succinate and acetate without lag whereas p-hydroxy benzoate, m-hydroxy benzoate and adipate were oxidised with lag (Figs.33,34,35).

(viii) Glucose-grown cells - Benzaldehyde, catechol, succinate and acetate were oxidised without lag whereas benzoate, p-hydroxy benzoate, m-hydroxy

Fig.24. Ability of m-hydroxy benzoate-grown cells to oxidize p-hydroxy benzoate, adipate and acetate. Each Warburg flask contained 4 mg dry weight equivalent of m-hydroxy benzoate-grown cells and 2 μ mole substrate.

A ... p-hydroxy benzoate

B ... Adipate

C ... Acetate

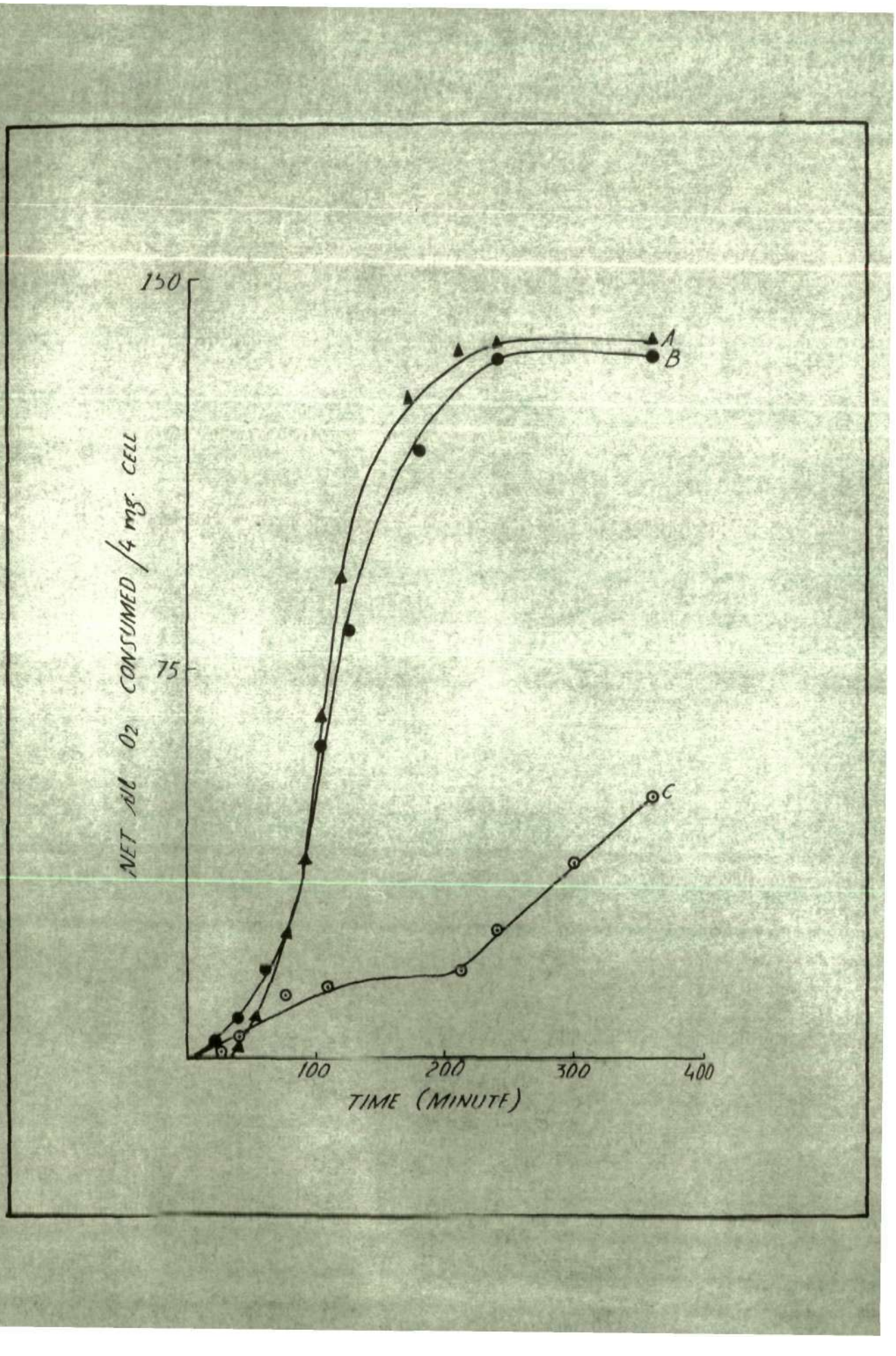

150
75
NET µl O2 CONSUMED /4 mg. CELL
A
B
C
100
200
300
400
TIME (MINUTE)

Fig.25. Ability of m-hydroxy benzoate-grown cells to oxidise m-hydroxy benzoate, succinate and protocatechuate. Each Warburg flask contained 4 mg dry weight equivalent of m-hydroxy benzoate-grown cells and 2 μ mole substrate.

A ... m-hydroxy benzoate

B ... Succinate

C ... Protocatechuate

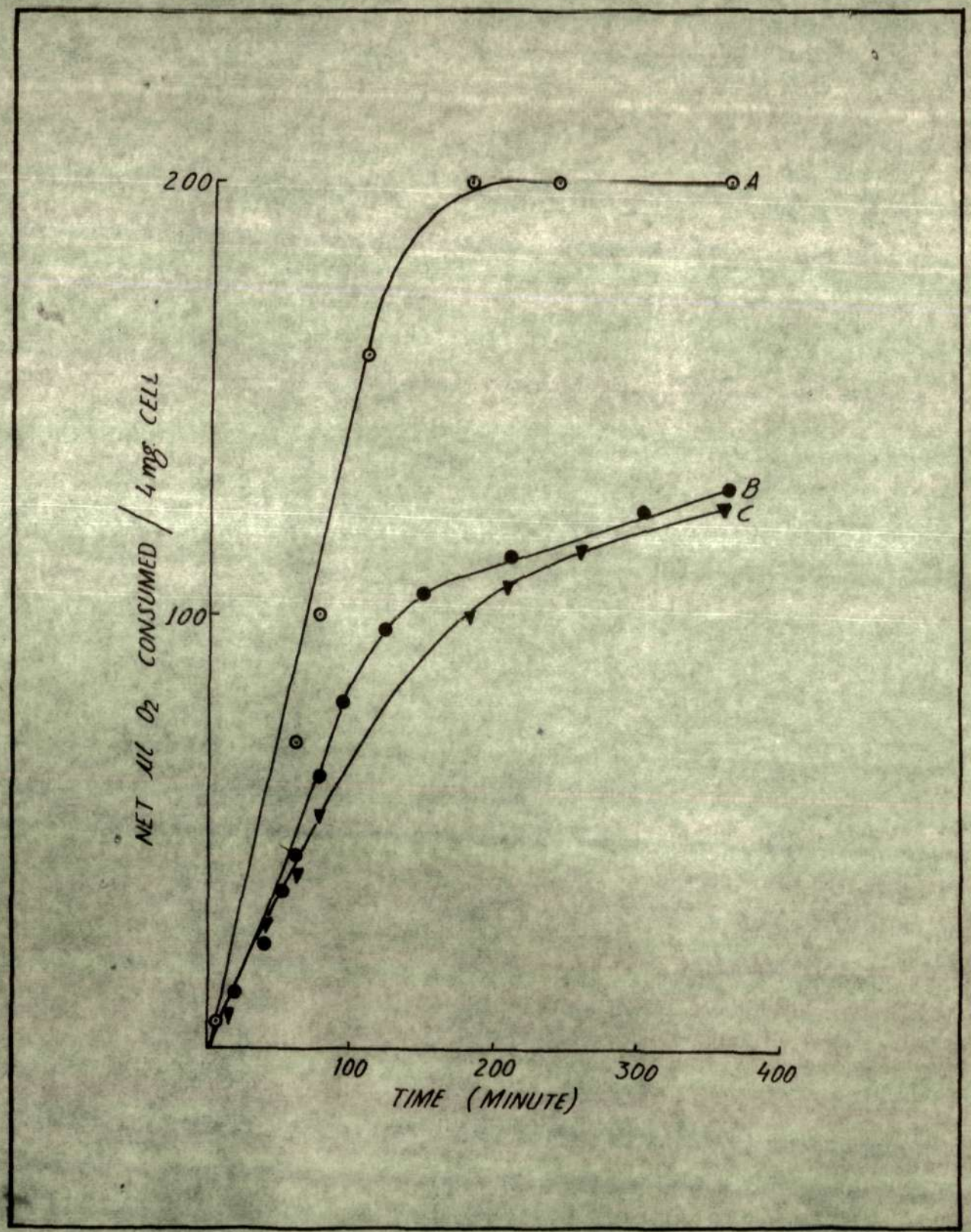

200
100
A
B
C
NET µl O2 CONSUMED / 4 mg. CELL
100
200
300
400
TIME (MINUTE)

Fig.26. Ability of m-hydroxy benzoate-grown cells to oxidize benzaldehyde, catechol, 2,4-dihydroxy benzaldehyde and benzoate. Each Warburg flask contained 4 mg dry weight equivalent of m-hydroxy benzoate-grown cells and 2 μ mole substrate.

A ... Benzaldehyde

B ... Catechol

C ... 2,4-dihydroxy benzaldehyde

D ... Benzoate

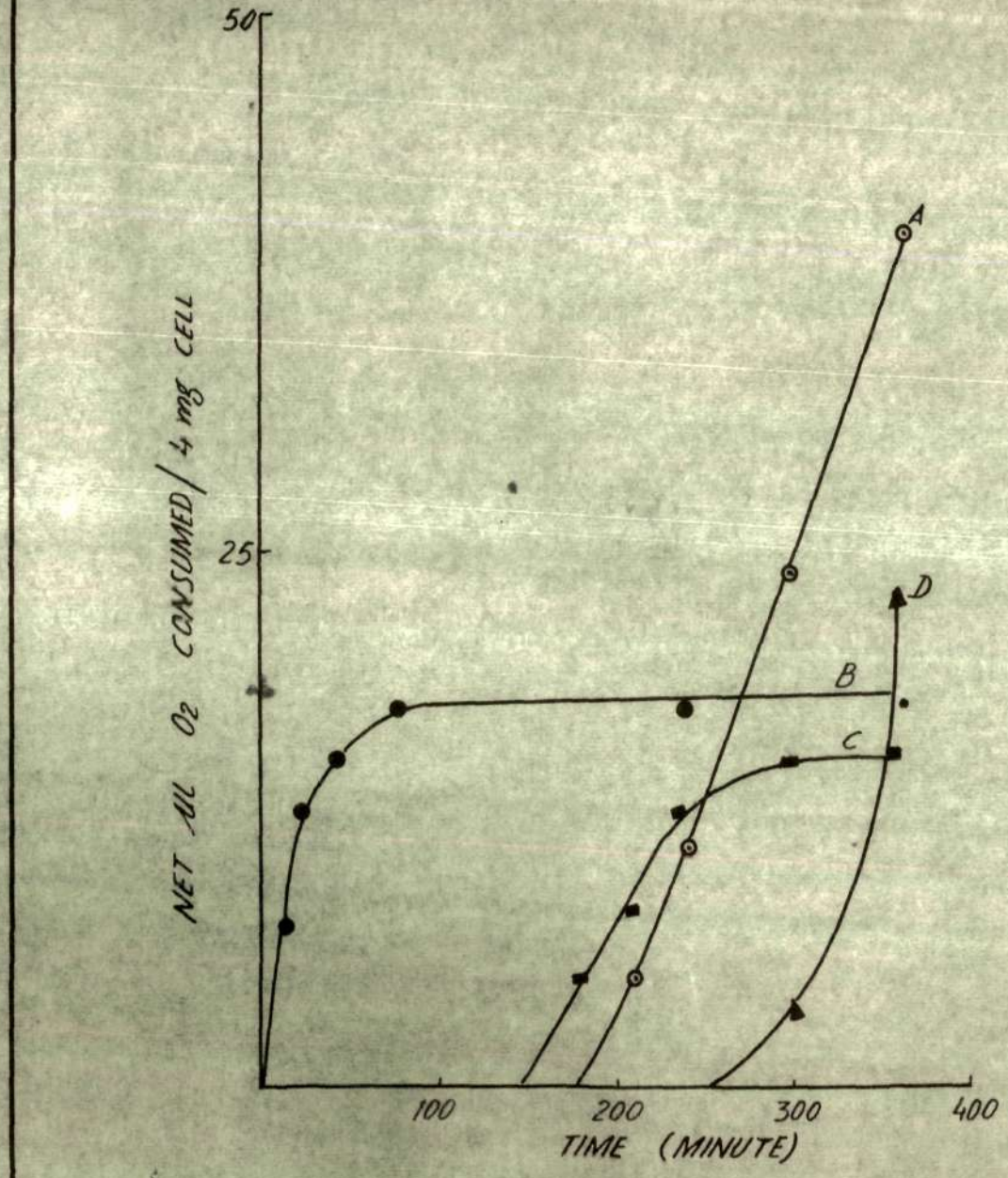

50
25
NET µL O2 CONSUMED / 4 mg CELL
A
B
C
D
100
200
300
400
TIME (MINUTE)

Fig.27. Ability of adipate-grown cells to oxidize protocatechuate, succinate and acetate. Each Warburg flask contained 9 mg dry weight equivalent of adipate-grown cells and 2 μ mole substrate.

A ... Protocatechuate

B ... Succinate

C ... Acetate

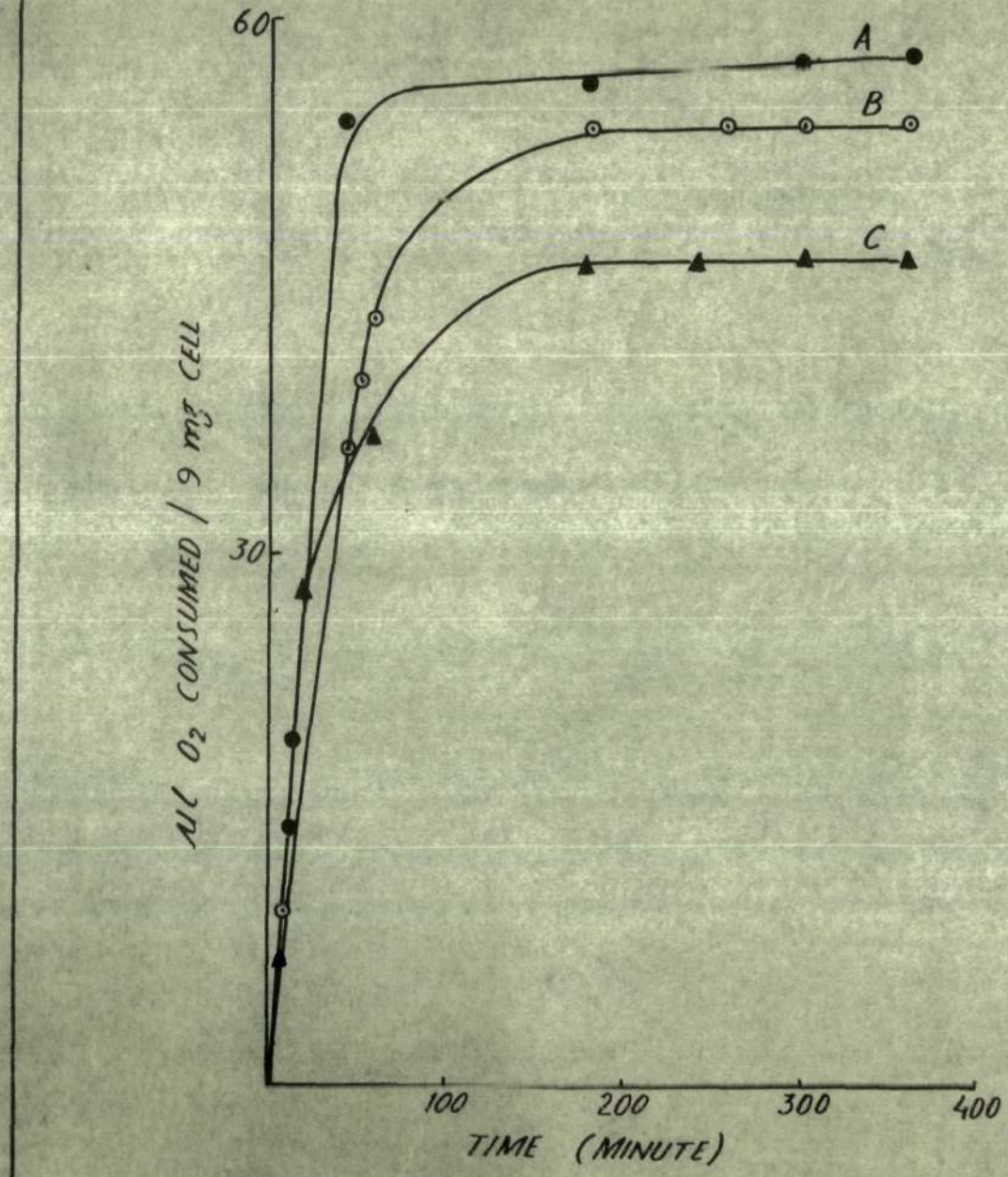

60
30
A
B
C
µl O2 CONSUMED / 9 mg CELL
100
200
300
400
TIME (MINUTE)

Fig.28. Ability of adipate-grown cells to oxidize m-hydroxy benzoate, benzoate and p-hydroxy benzoate. Each Warburg flask contained 9 mg dry weight equivalent of adipate-grown cells and 2 μ mole substrate.

A ... m-hydroxy benzoate

B ... Benzoate

C ... p-hydroxy benzoate

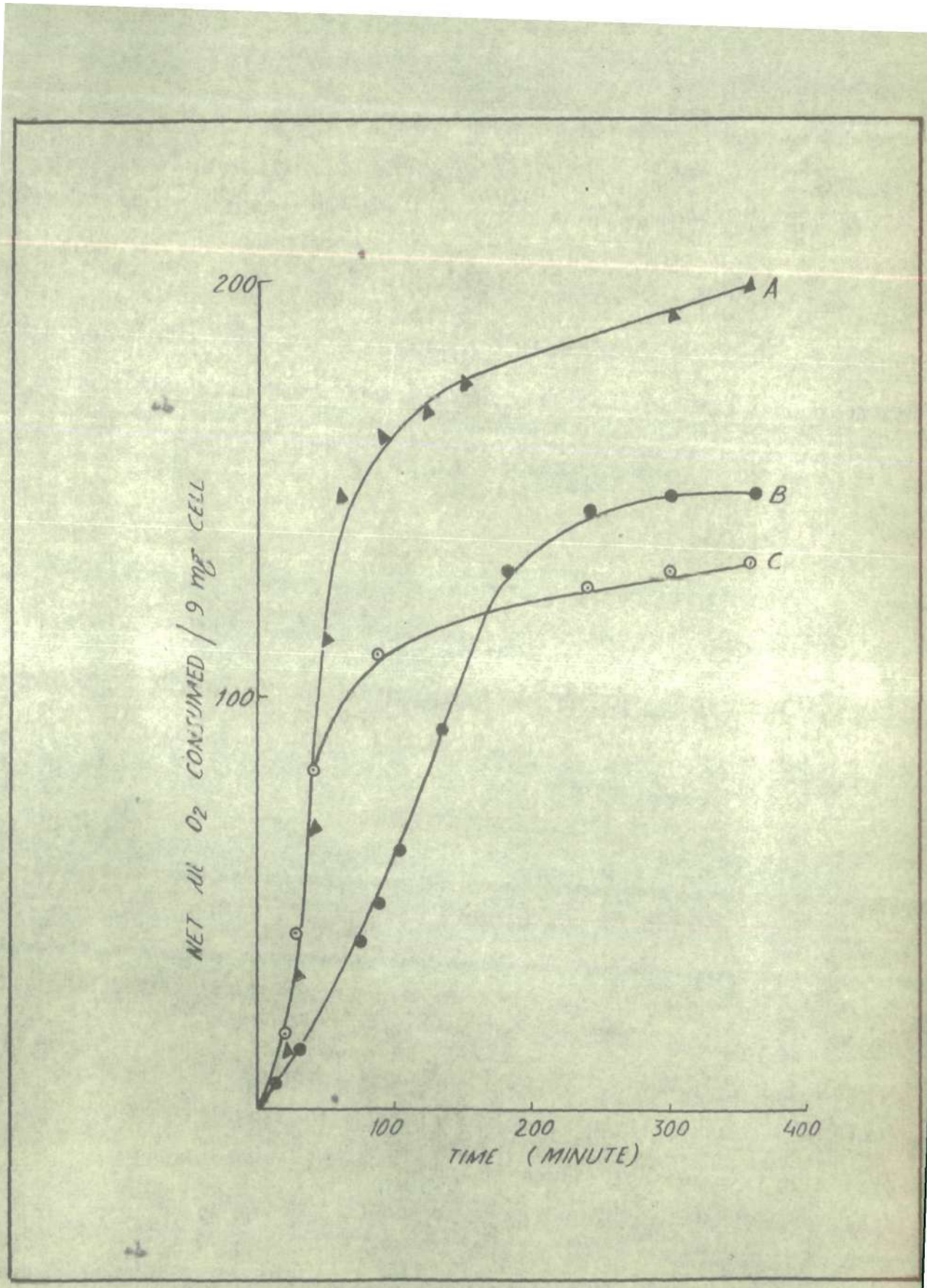

200
100
NET µl O2 CONSUMED / 9 mg CELL
A
B
C
100
200
300
400
TIME (MINUTE)

Fig.29. Ability of adipate-grown cells to oxidize adipate, benzaldehyde and 2,4-dihydroxy benzaldehyde. Each Warburg flask contained 9 mg dry weight equivalent of adipate-grown cells and 2 μ mole substrate.

A ... Adipate

B ... Benzaldehyde

C ... 2,4-dihydroxy benzaldehyde

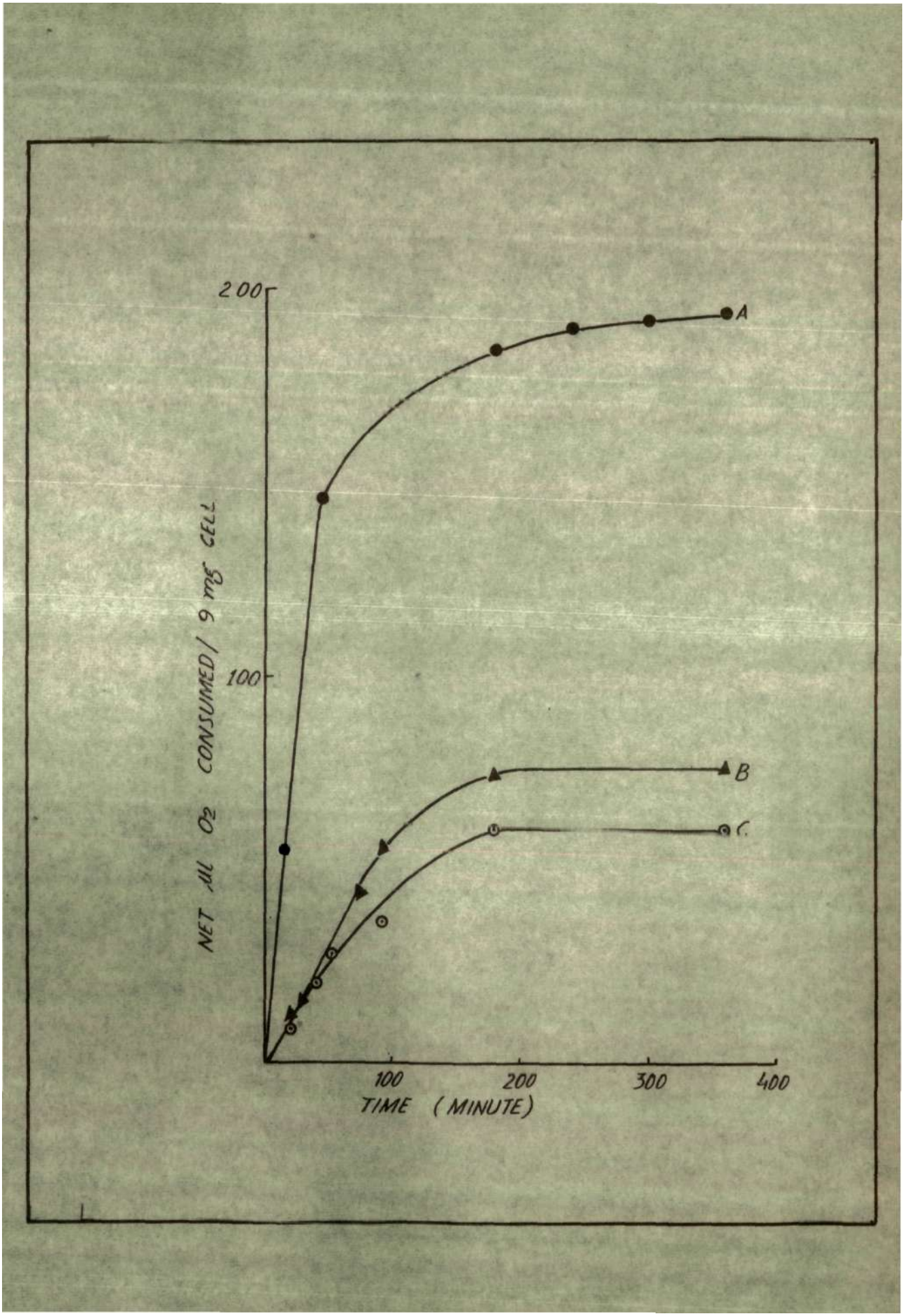

200
100
NET µl O2 CONSUMED/ 9 mg CELL
A
B
C
100
200
300
400
TIME (MINUTE)

Fig.30. Ability of acetate-grown cells to oxidize benzoate, adipate, succinate and acetate. Each Warburg flask contained 3 mg dry weight equivalent of acetate-grown cells and 2 μ mole substrate.

A ... Benzoate

B ... Adipate

C ... Succinate

D ... Acetate

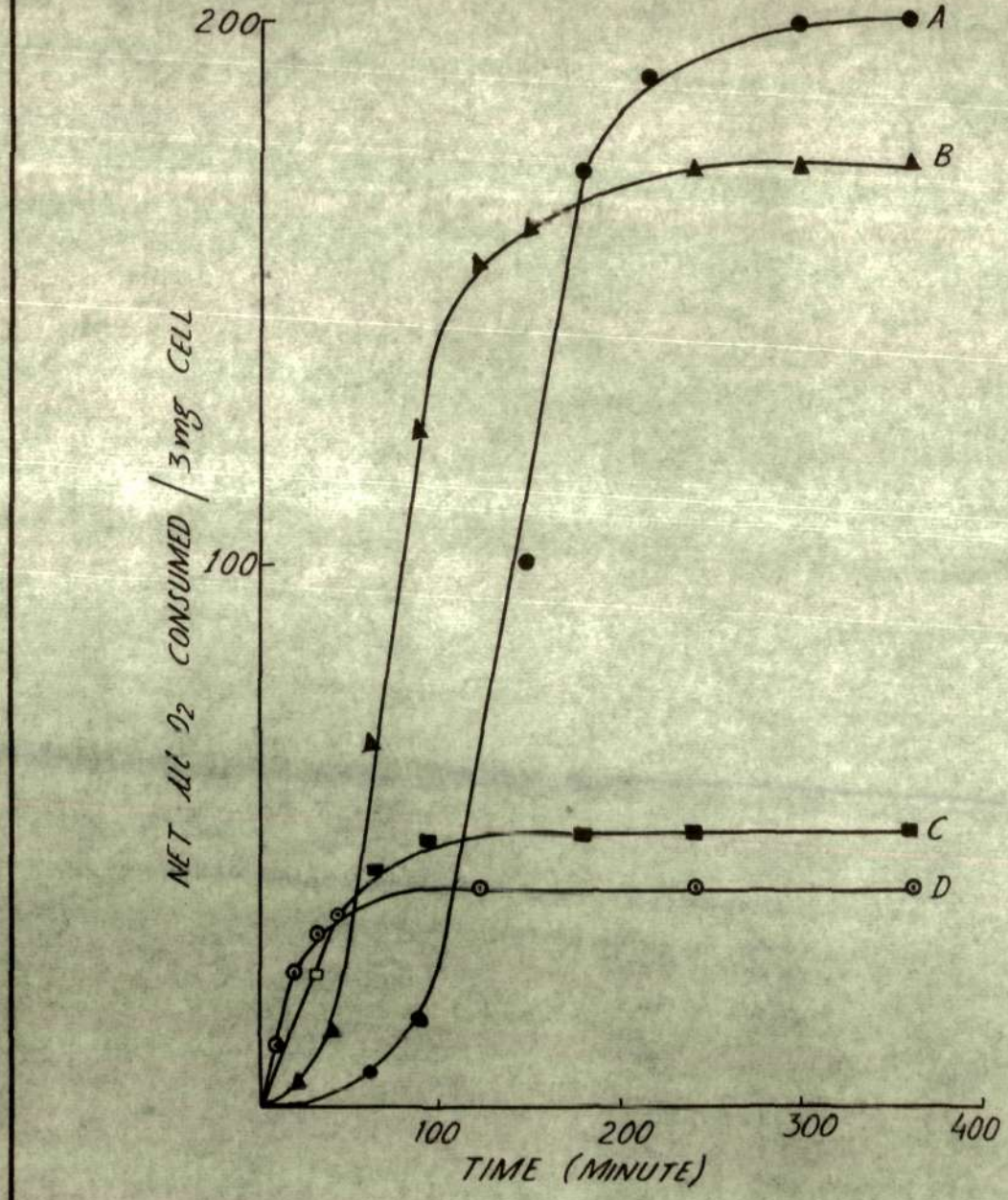

200
100
NET µl O_2 CONSUMED / 3mg CELL
A
B
C
D
100
200
300
400
TIME (MINUTE)

Fig.31. Ability of acetate-grown cells to oxidize 2,4-dihydroxy benzaldehyde, benzaldehyde and catechol. Each Warburg flask contained 3 mg dry weight equivalent of acetate-grown cells and 2 μ mole substrate.

A ... 2,4-dihydroxy benzaldehyde

B ... Benzaldehyde

C ... Catechol

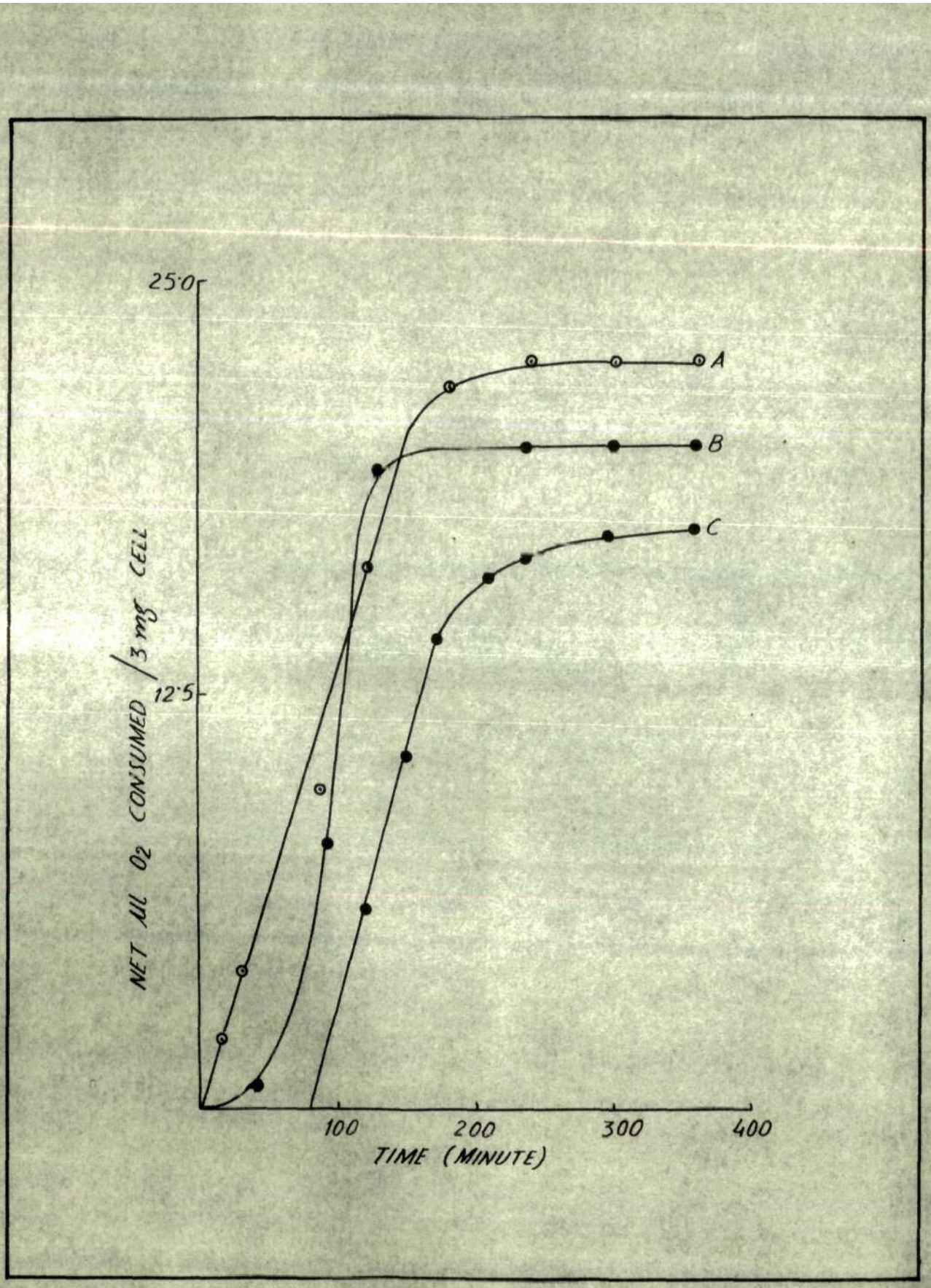

25·0
12·5
NET µl O2 CONSUMED /3 mg CELL
A
B
C
100
200
300
400
TIME (MINUTE)

Fig.32. Ability of acetate-grown cells to oxidize p-hydroxy benzoate, m-hydroxy benzoate and protocatechuate. Each Warburg flask contained 3 mg dry weight equivalent of acetate-grown cells and 2 μ mole substrate.

A ... p-hydroxy benzoate

B ... m-hydroxy benzoate

C ... Protocatechuate

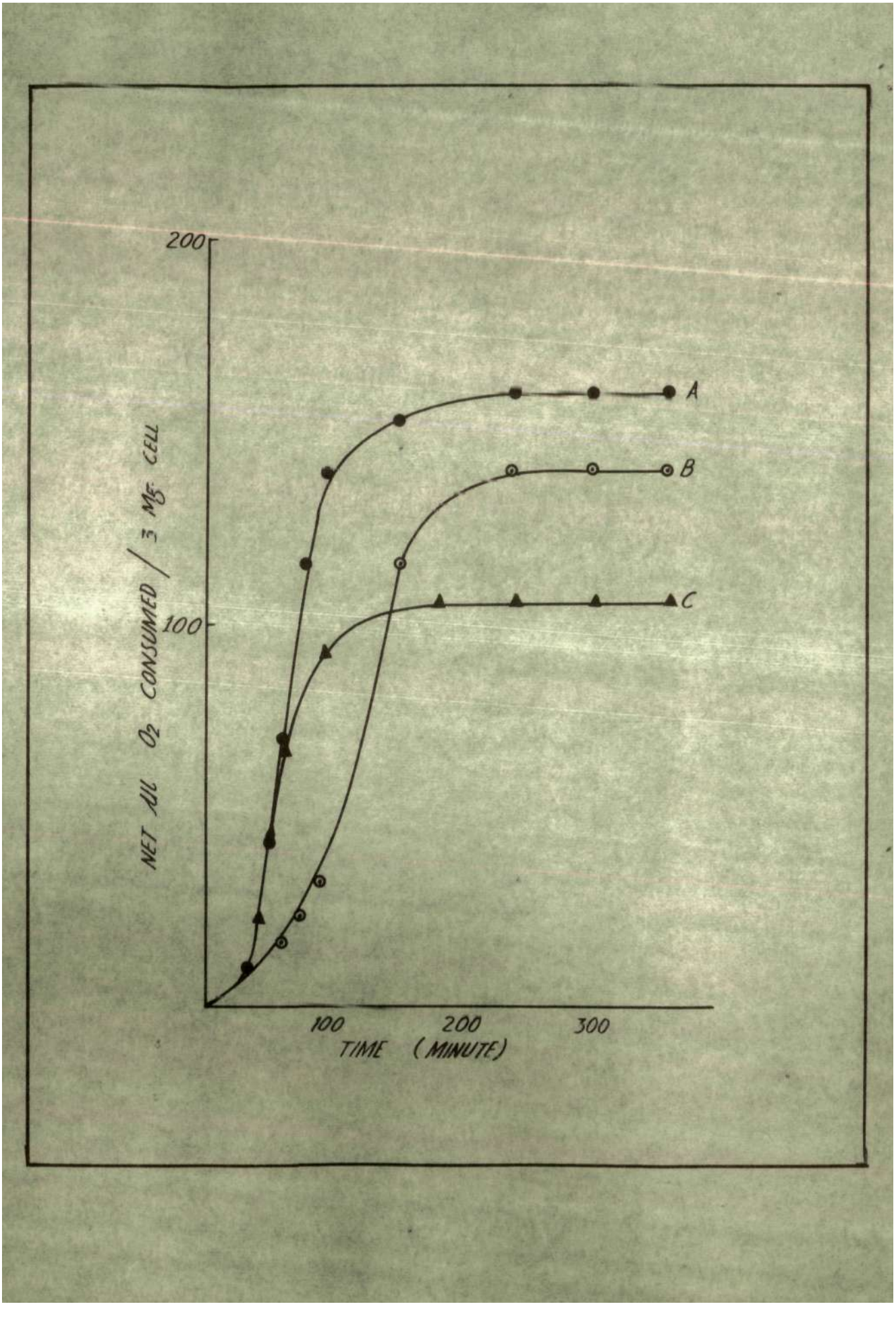

200
100
NET µl O2 CONSUMED / 3 MG. CELL
A
B
C
100
200
300
TIME (MINUTE)

Fig.33. Ability of succinate-grown cells to oxidize catechol, 2,4-dihydroxy benzaldehyde, benzoate and benzaldehyde. Each Warburg flask contained 8 mg dry weight equivalent of succinate-grown cells and 2 μ mole substrate.

A ... Catechol

B ... 2,4-dihydroxy benzaldehyde

C ... Benzoate

D ... Benzaldehyde

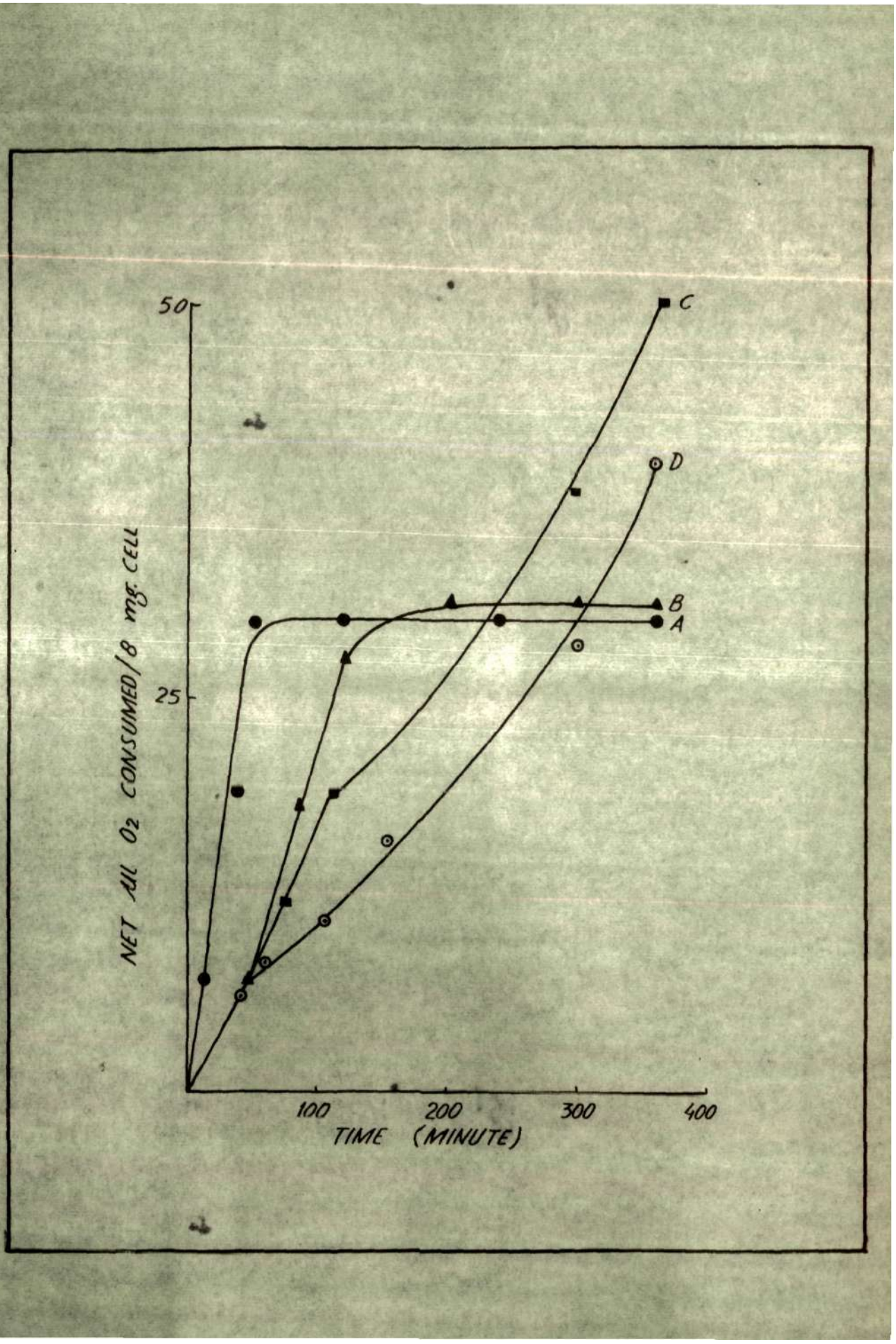

50
25
C
D
B
A
NET µl O2 CONSUMED/8 mg. CELL
100
200
300
400
TIME (MINUTE)

Fig.34. Ability of succinate-grown cells to oxidize m-hydroxy benzoate, p-hydroxy benzoate and adipate. Each Warburg flask contained 8 mg dry weight equivalent of succinate-grown cells and 2 μ mole substrate.

A ... m-hydroxy benzoate

B ... Adipate

C ... p-hydroxy benzoate

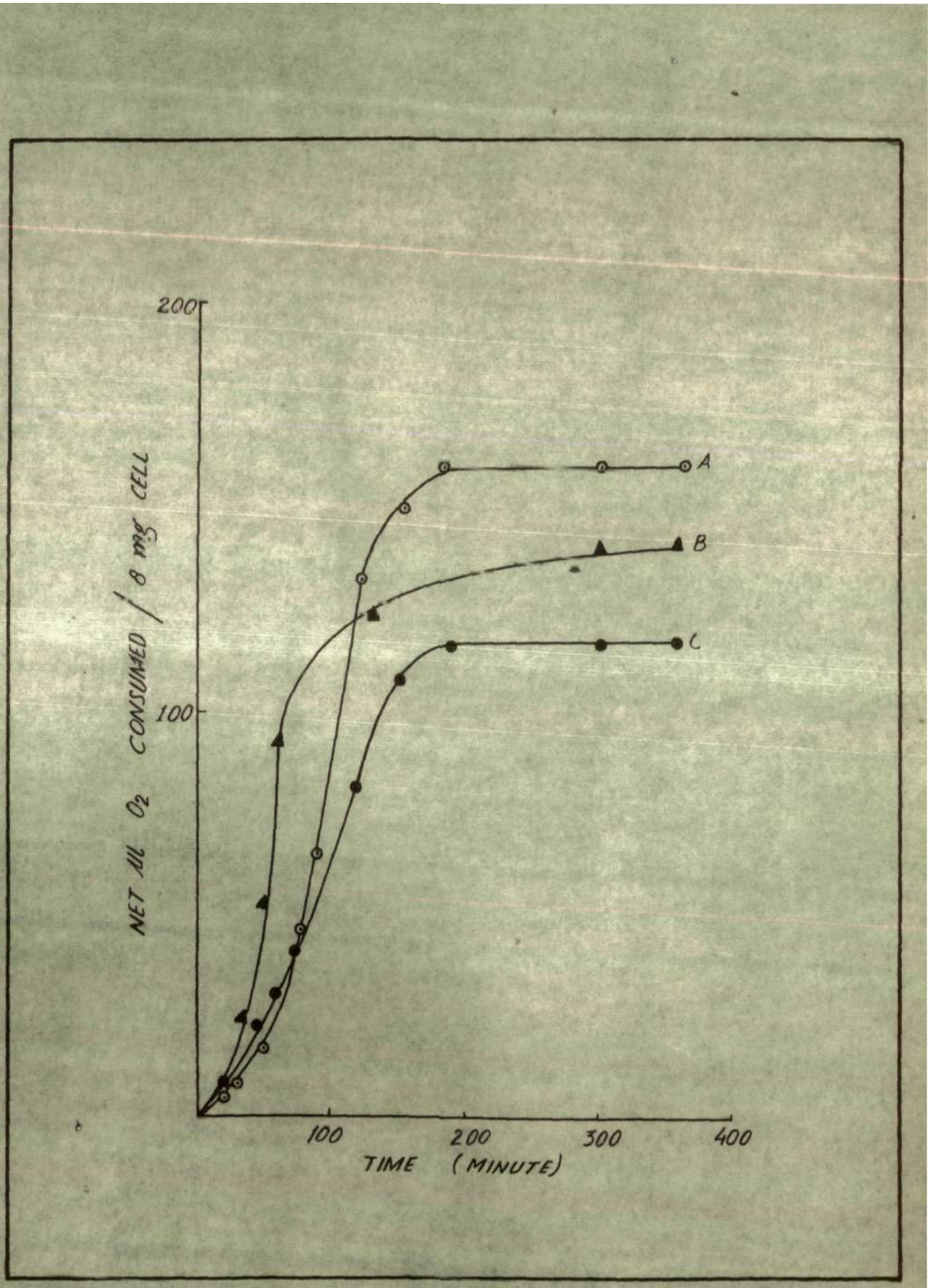
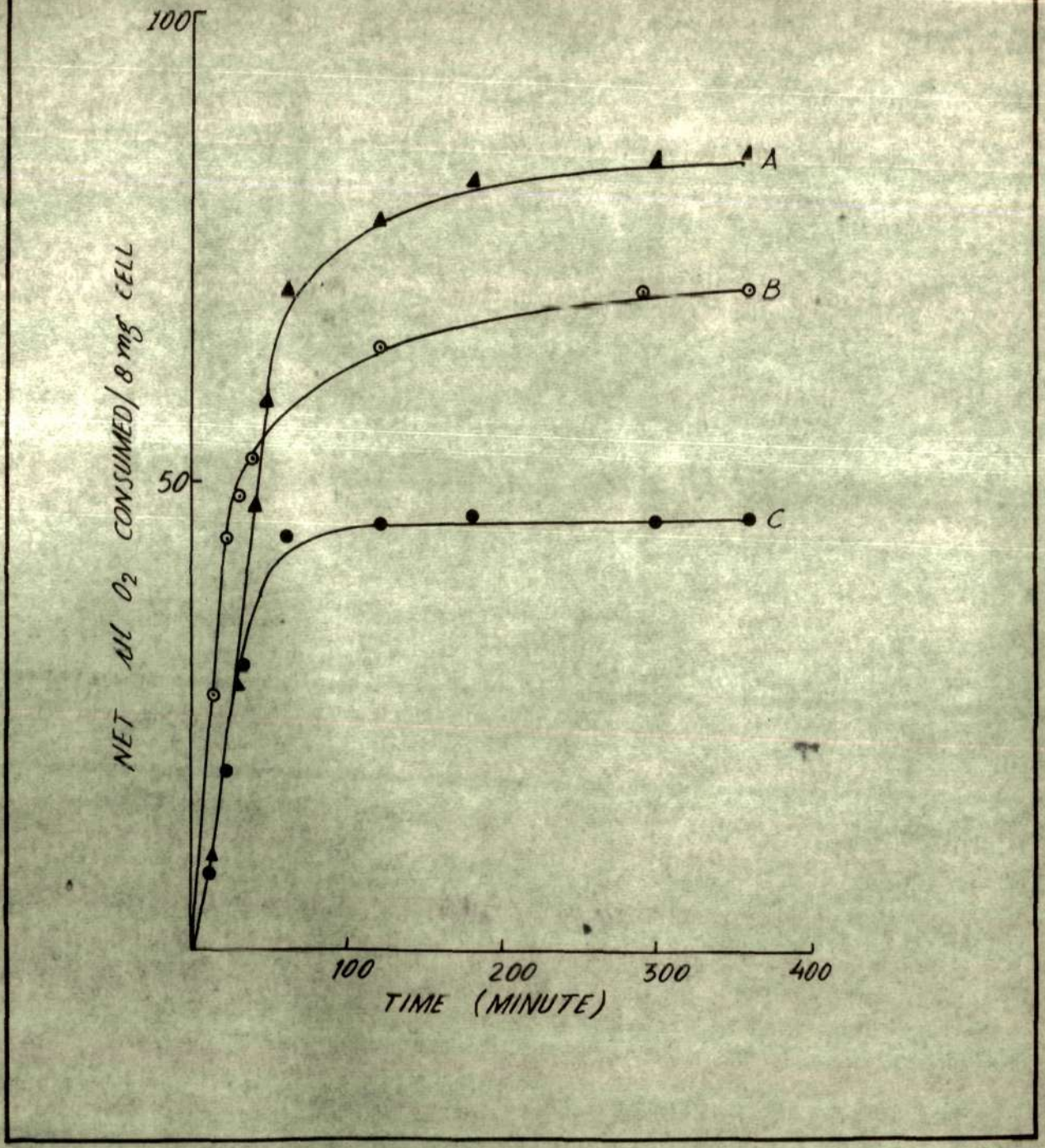

200
100
NET µl O2 CONSUMED / 8 mg CELL
A
B
C
100
200
300
400
TIME (MINUTE)

Fig.35. Ability of succinate-grown cells to oxidize protocatechuate, succinate and acetate. Each Warburg flask contained 8 mg dry weight equivalent of succinate-grown cells and 2 μ mole substrate.

A ... Protocatechuate

B ... Succinate

C ... Acetate


100
50
NET μl O_2 CONSUMED/8 mg CELL
A
B
C
100
200
300
400
TIME (MINUTE)

benzoate , protocatechuate and adipate were oxidised with lag. In comparison to benzaldehyde-grown cells the oxygen consumptions with m-hydroxy benzoate and benzaldehyde were very poor. (Figs. 36,37,38). In contrast to earlier observation with benzaldehyde-grown cells protein synthesis inhibitors had a marked effect on these cells. In presence of 0.3 mM puromycin or 10 mM o-nitrobenzoic acid the rate of oxidation of benzaldehyde was not effected but the stoichiometry of oxygen uptake increased (Figs. 39,41). The rate of oxidation of p-hydroxy benzoate decreased but the total oxygen uptake increased (Figs.40,42). m-hydroxy benzoate was not oxidised at all in presence of these inhibitors. In presence of 0.5 mM chloramphenicol the rate of oxidation as well as total oxygen uptake of benzaldehyde decreased (Fig.43), p-hydroxy benzoate and m-hydroxy benzoate were not oxidised at all because their oxidation was equal to endogenous (Fig.44).

In all the above cases bacteria exposed to the substrate, on which they had been grown, gave an immediate oxygen uptake, showing that the appropriate

Fig.36. Ability of glucose-grown cells to oxidize succinate, catechol and acetate. Each Warburg flask contained 4 mg dry weight equivalent of glucose-grown cells and 2 μ mole substrate.

A ... Succinate

B ... Catechol

C ... Acetate

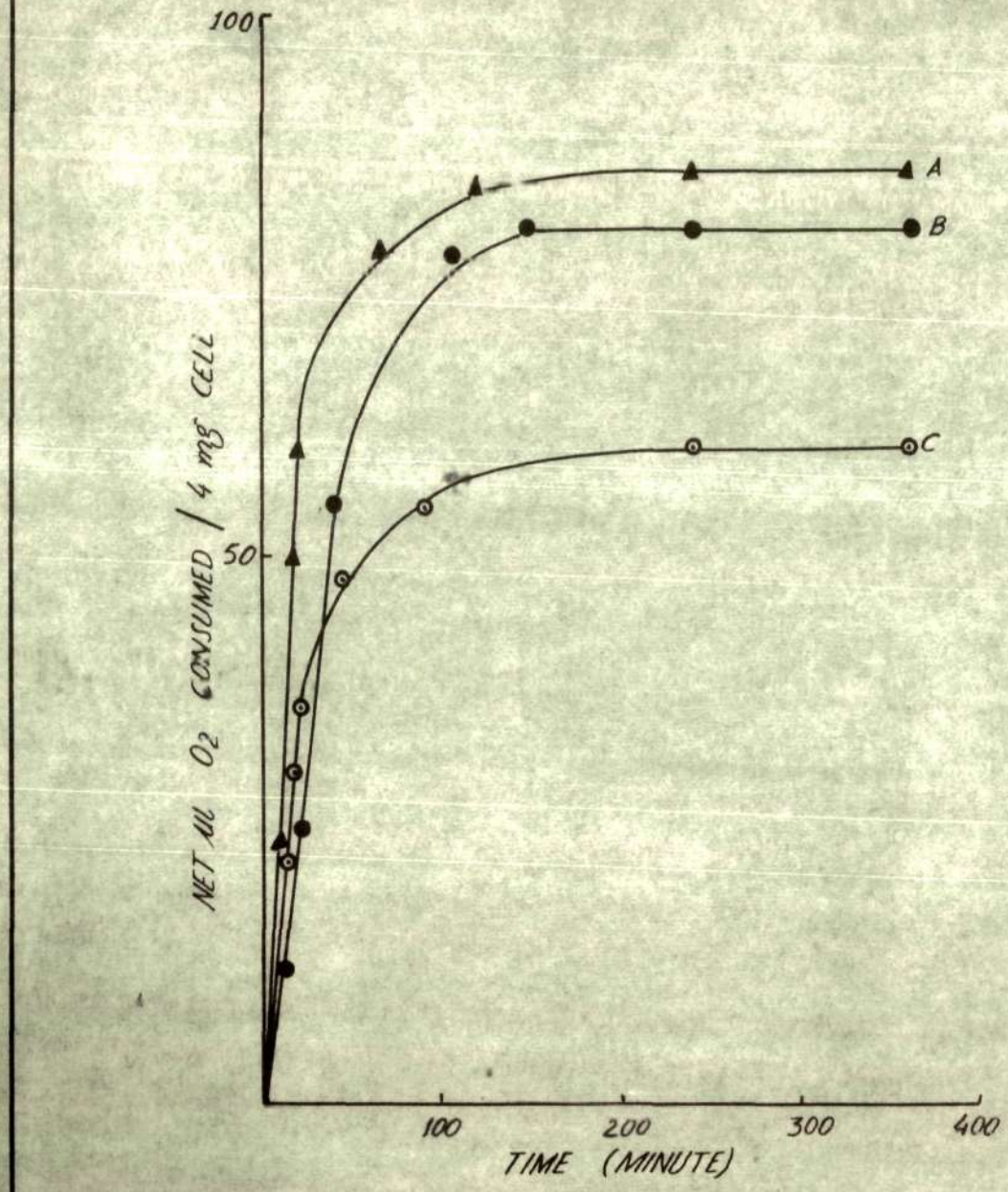

100
50
NET μl O2 CONSUMED / 4 mg CELL
A
B
C
100
200
300
400
TIME (MINUTE)

Fig.37. Ability of glucose-grown cells to oxidize benzoate, p-hydroxy benzoate and protocatechuate. Each Warburg flask contained 4 mg dry weight equivalent of glucose-grown cells and 2 μ mole substrate.

A ... Benzoate

B ... p-hydroxy benzoate

C ... Protocatechuate

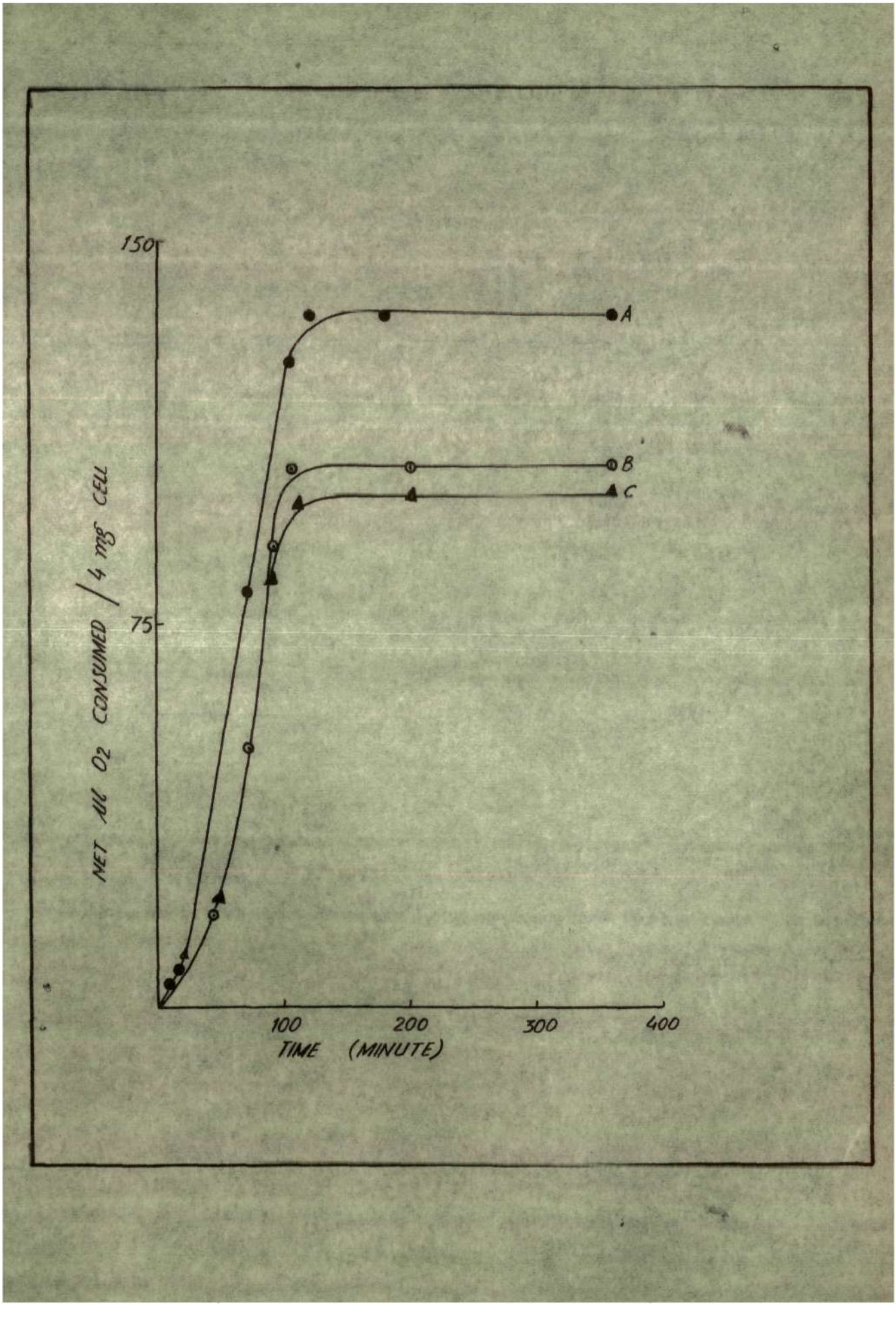

150
75
NET µl O2 CONSUMED / 4 mg CELL
A
B
C
100
200
300
400
TIME (MINUTE)

Fig.38. Ability of glucose-grown cells to oxidize adipate, benzaldehyde and m-hydroxy benzoate. Each Warburg flask contained 4 mg dry weight equivalent of glucose-grown cells and 2 μ mole substrate.

A ... Adipate

B ... Benzaldehyde

C ... m-hydroxy benzoate

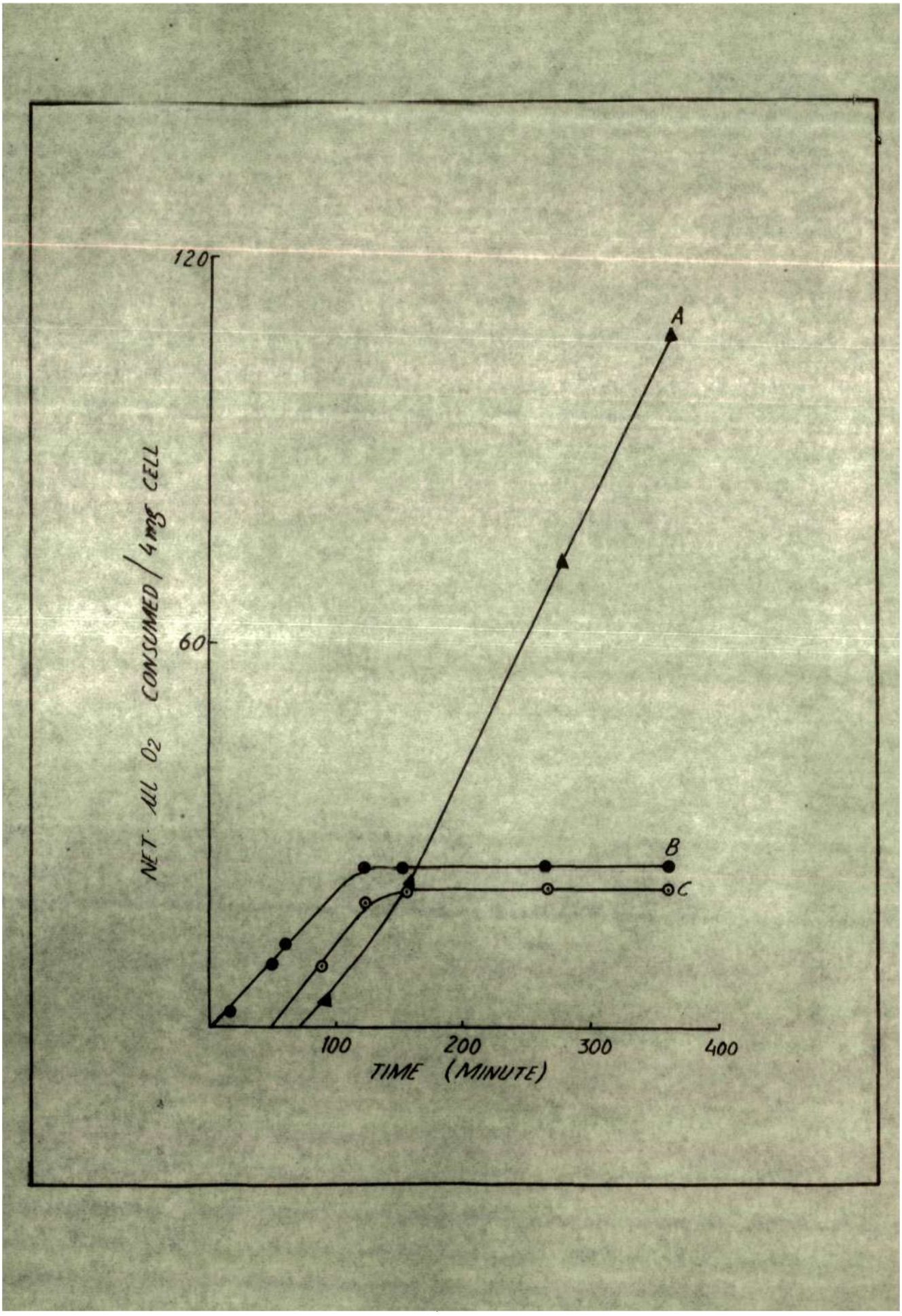

120
60
NET µL O2 CONSUMED / 4 mg CELL
A
B
C
100
200
300
400
TIME (MINUTE)

Fig.39. Ability of glucose-grown cells to oxidize benzaldehyde in presence of puromycin. Each Warburg flask contained 4 mg dry weight equivalent of glucose-grown cells, 0.6 μ mole puromycin and 2 μ mole benzaldehyde. The endogenous flask niether contained substrate nor puromycin.

A ... Benzaldehyde + Puromycin

B ... Benzaldehyde

C ... Puromycin

D ... Endogenous

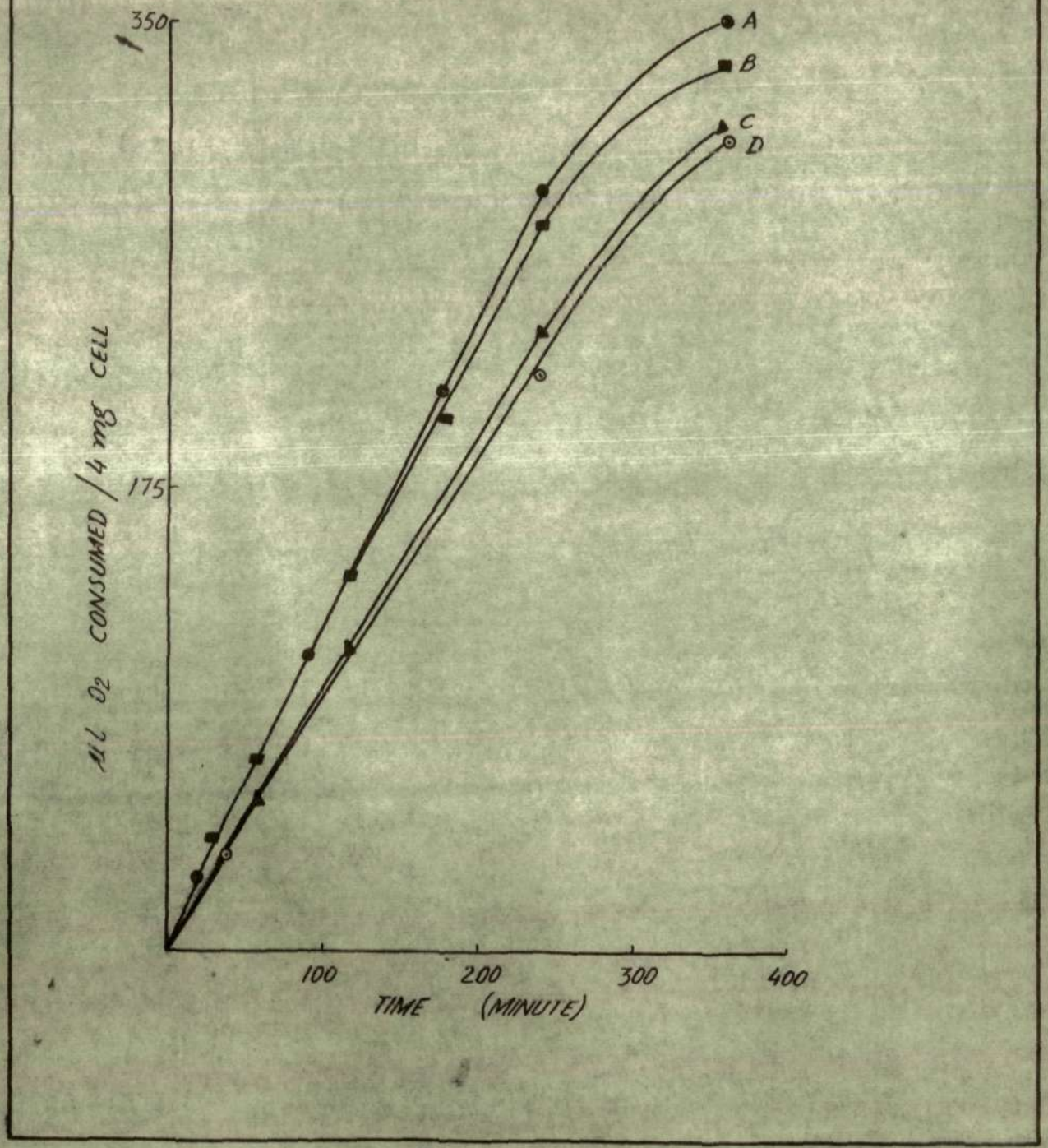

350
175
µl O2 CONSUMED / 4 mg CELL
A
B
C
D
100
200
300
400
TIME (MINUTE)

Fig.40. Ability of glucose-grown cells to oxidize p-hydroxy benzoate in presence of puromycin. Each Warburg flask contained 4 mg dry weight equivalent of glucose-grown cells, 0.6 μ mole puromycin and 2 μ mole p-hydroxy benzoate. The endogenous flask niether contained substrate nor puromycin.

A ... p-hydroxy benzoate + Puromycin

B ... p-hydroxy benzoate

C ... Puromycin

D ... Endogenous

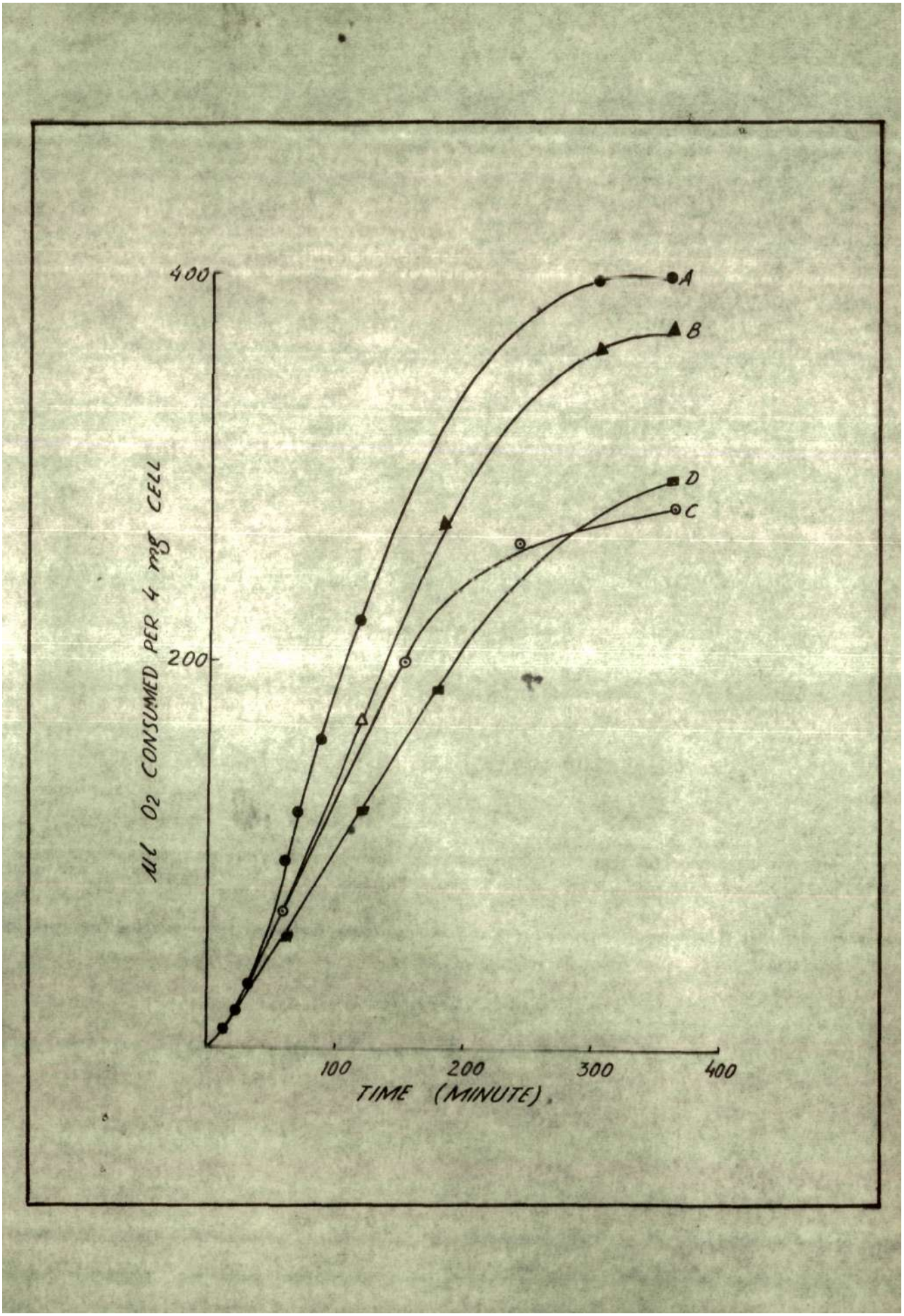

400
200
A
B
D
C
µl O2 CONSUMED PER 4 mg CELL
100
200
300
400
TIME (MINUTE).

Fig. 41. Ability of glucose-grown cells to oxidize benzaldehyde in presence of o-nitrobenzoic acid. Each Warburg flask contained 4 mg dry weight equivalent of glucose-grown cells, 20 μ mole o-nitrobenzoic acid and 2 μ mole benzaldehyde. The endogenous flask niether contained substrate nor o-nitrobenzoic acid.

A ... Benzaldehyde + o-nitrobenzoic acid

B ... Benzaldehyde

C ... o-nitrobenzoic acid

D ... Endogenous

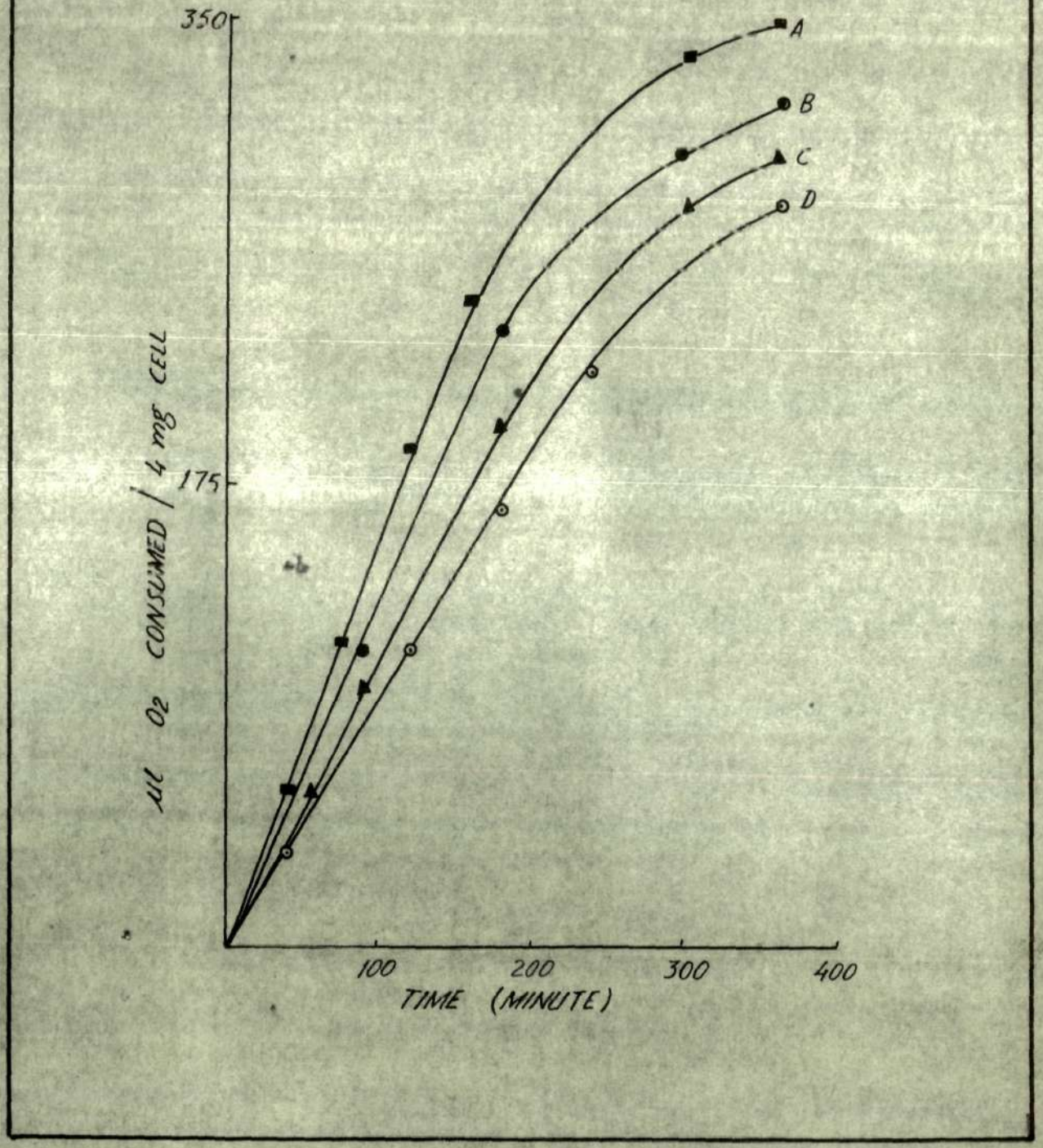

350
175
μl O2 CONSUMED / 4 mg CELL
A
B
C
D
100
200
300
400
TIME (MINUTE)

Fig.42. Ability of glucose-grown cells to oxidize p-hydroxy benzoate in presence of o-nitrobenzoic acid. Each Warburg flask contained 4 mg dry weight equivalent of glucose-grown cells, 20 μ mole o-nitrobenzoic acid and 2 μ mole p-hydroxy benzoate. The endogenous flask niether contained substrate nor o-nitrobenzoic acid.

A ... p-hydroxy benzoate + o-nitrobenzoic acid

B ... p-hydroxy benzoate

C ... o-nitrobenzoic acid

D ... Endogenous

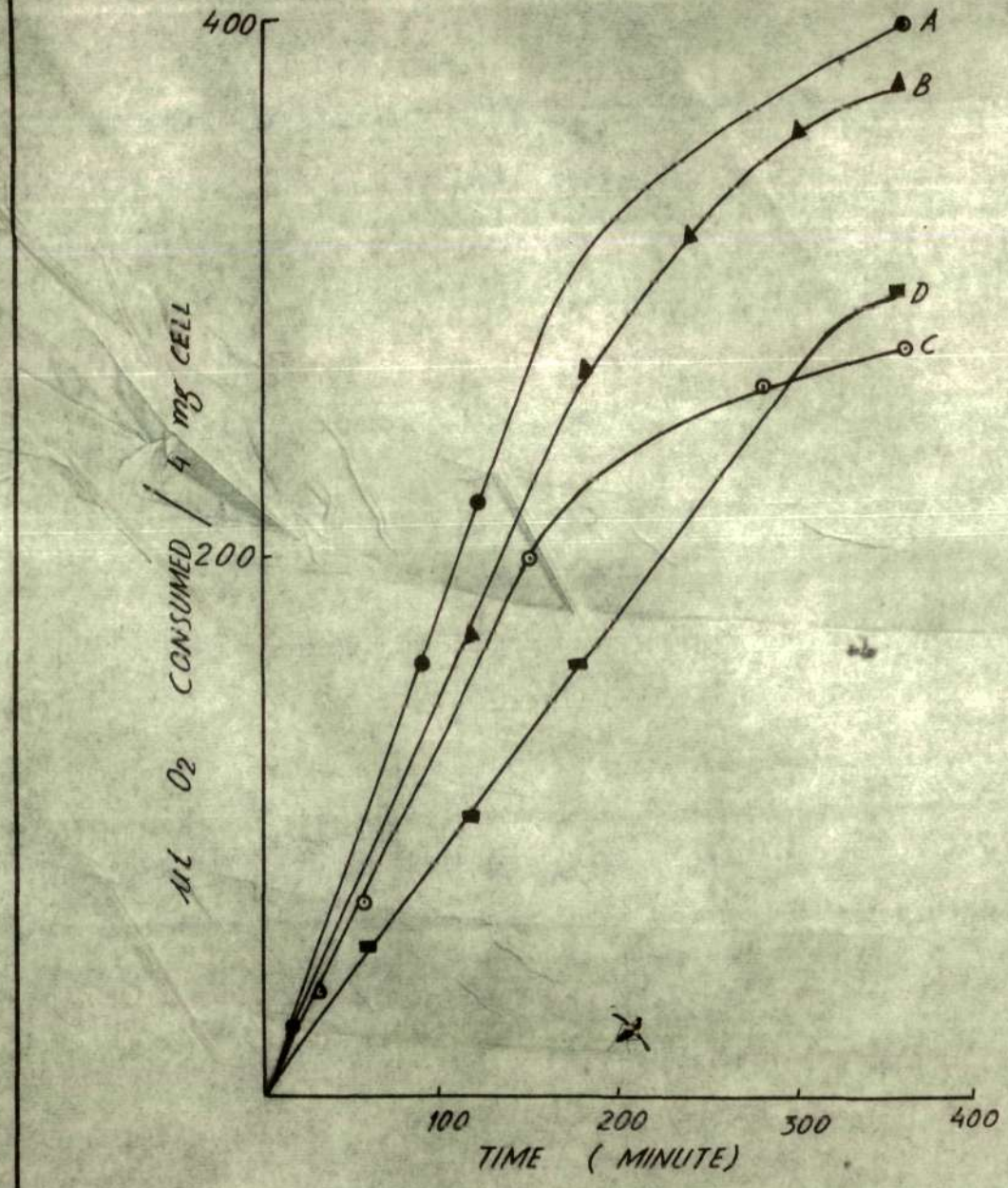

400
200
A
B
D
C
µl O2 CONSUMED / 4 mg CELL
100
200
300
400
TIME (MINUTE)

Fig.43. Ability of glucose-grown cells to oxidize benzaldehyde in presence of chloramphenicol. Each Warburg flask contained 4 mg dry weight equivalent of glucose-grown cells, 1 μ mole chloramphenicol and 2 μ mole benzaldehyde. The endogenous flask niether contained substrate nor chloramphenical.

A ... Benzaldehyde

B ... Endogenous

C ... Benzaldehyde + Chloramphenicol

D ... Chloramphenicol

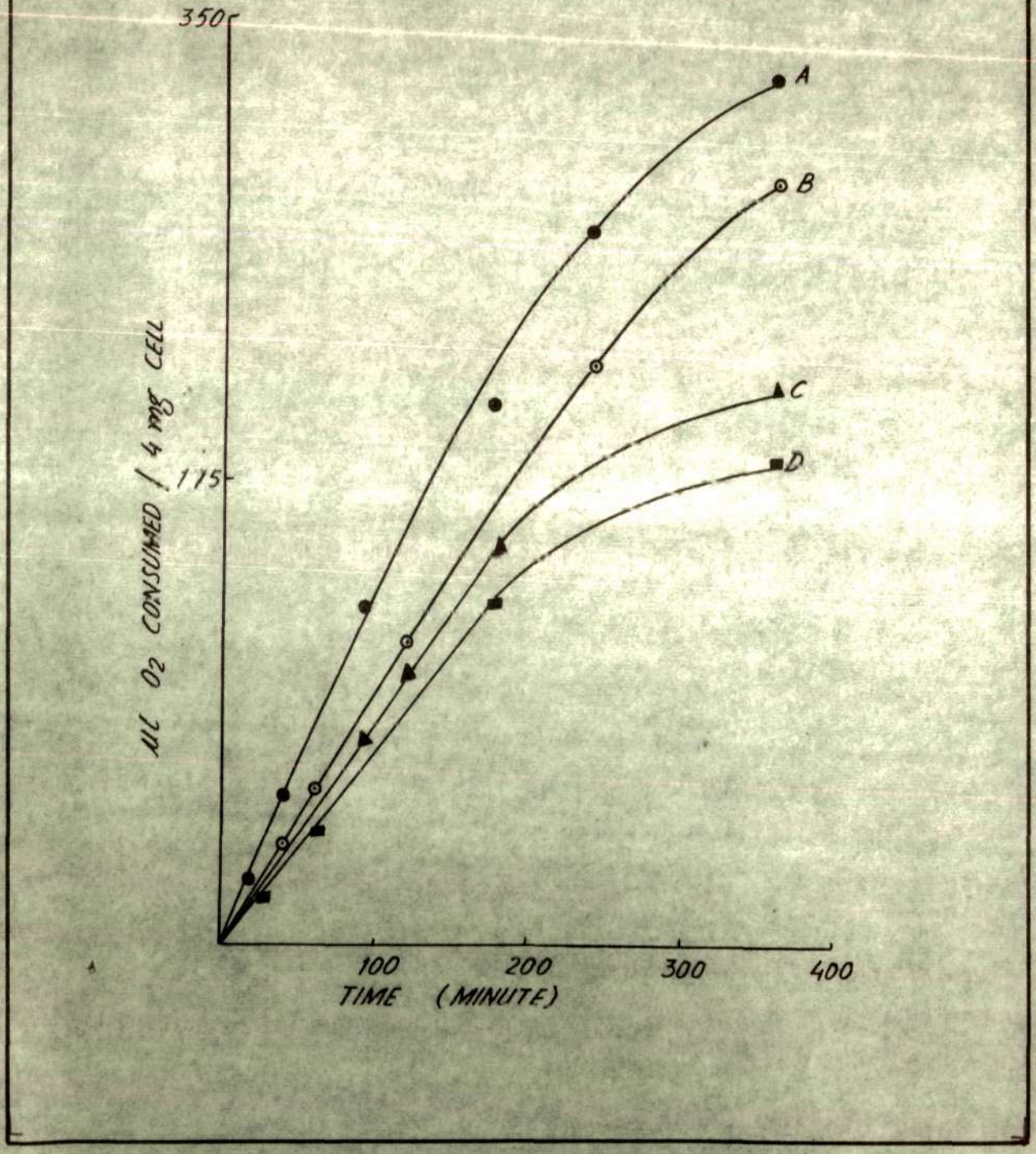

350
175
µl O2 CONSUMED / 4 mg CELL
A
B
C
D
100
200
300
400
TIME (MINUTE)

Fig.44. Ability of glucose-grown cells to oxidize p-hydroxy benzoate and m-hydroxy benzoate in presence of chloramphenicol. Each Warburg flask contained 4 mg dry weight equivalent of glucose-grown cells, 1 μ mole chloramphenicol and 2 μ mole substrate. The endogenous flask niether contained substrate nor chloramphenicol.

A ... p-hydroxy benzoate

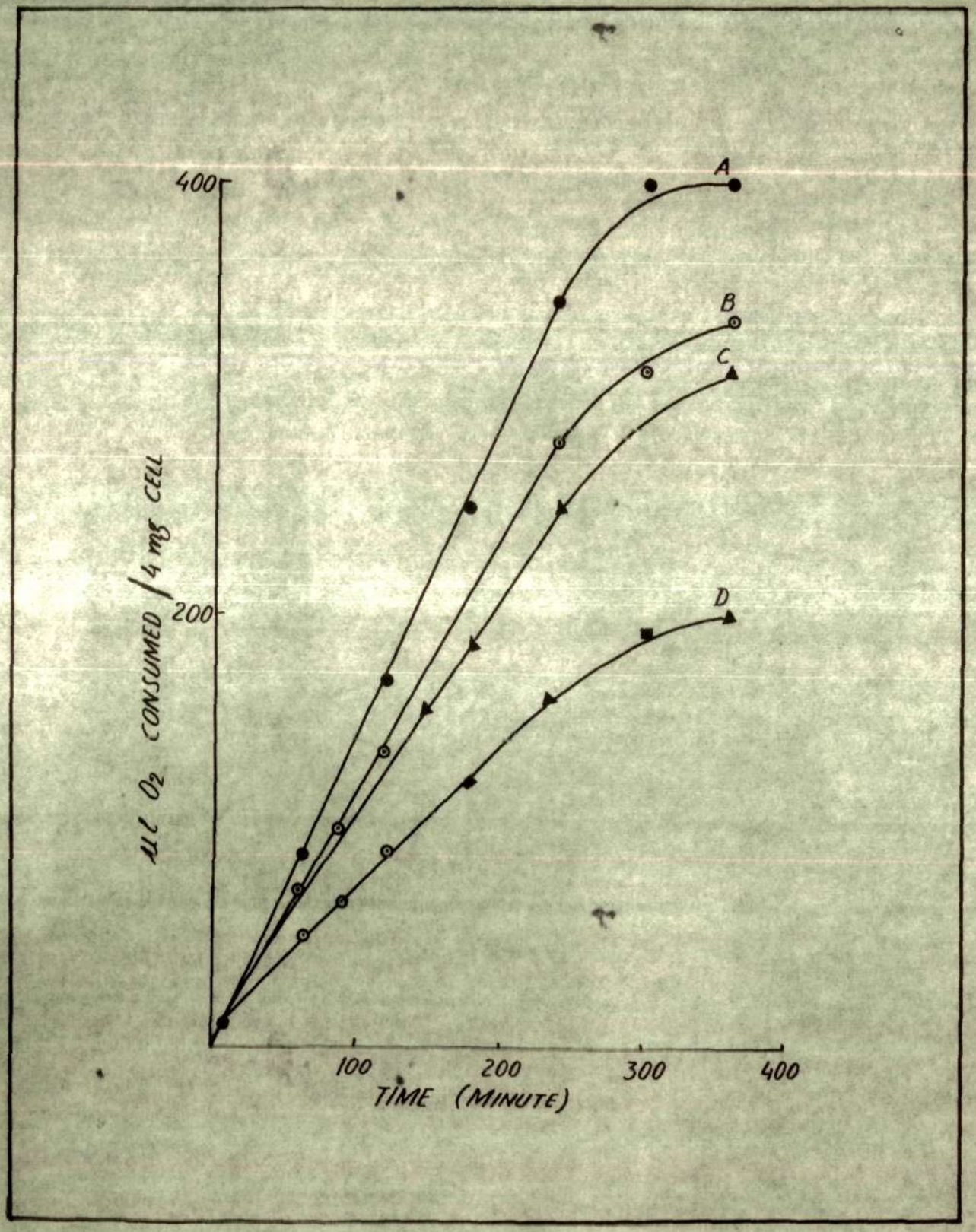
B ... m-hydroxy benzoate

C ... Endogenous

D ... Chloramphenicol

⊙ ... p-hydroxy benzoate + chloramphenicol

▲ ... m-hydroxy benzoate + chloramphenicol


400
200
A
B
C
D
µl O2 CONSUMED / 4 mg CELL
100
200
300
400
TIME (MINUTE)

enzymes had survived the preparation procedures. The types of oxygen uptake recorded for benzaldehyde-grown cells showed four patterns. First, as exmeplified by benzoate, catechol, acetate and protocatechuate (Fig.5), an immediate and rapid oxygen uptake which came to completion at about 50% of the oxygen consumption required for the complete oxidation of the substrate. Presumably the balance of 50% represented oxidative assimilation (Clifton, 1946). The second pattern (as shown by benzaldehyde, 2,4-dihydroxy benzaldehyde, salicylaldehyde and succinate in Fig.6) was an immediate but limited oxygen uptake. The thrid pattern of oxygen consumption was a lag followed by an increase in oxygen uptake (as shown by p-hydroxy benzoate. and adipate in Fig.7). The fourth pattern was a lag followed by an incomplete oxidation of the substrate as shown by m-hydroxy benzoate (Fig.7). The lag varied from 20 -150 minutes. The data obtained are summarized in Table X.

B. Studies with the Cell-free Extract - The cells of the benzaldehyde broth harvested in the lag phase were washed twice with equal volume of ice cold double distilled water. About 100 mg equivalent to dry weight

TABLE - X

Pattern of Oxygen Utilisation obtained with a number of challenge substrates when Incubated with Resting cell suspension of Achromobacter sp.

The organism was grown on each compound, in turn harvested, washed and the ability of the organism to oxidise various substrates estimated manometrically (Figs. 5 to 44). The values represent the initial rates of oxygen consumption (µ moles O2 / hr /mg dry cell equivalent / 2 µ moles substrate) with the endogenous rates substracted. The values in parenthesis represent the percent oxidation of substrate as calculated by conventional method (Dawes , 1962).

U = Immediate and complete utilization of O_2

L = lag followed by complete utilization

I-U = Immediate, incomplete O_2 uptake

I-L = lag followed by incomplete utilization

Challenge Substrate	Benzaldehyde	Benzoate	p-hyd. benzoate	m-hyd. benzoate	Adipate	Acetate	Succinate	Glucose
Benzaldehyde	I-1.43-U (22.9)	I-0.72-U (8.5)	I-L (14.0)	I-L (10.6)	I-0.15-U (20.8)	I-L (5.6)	I-0.05-U (10.6)	I-0.09-U (6.9)
Benzoate	U-2.06 (58.0)	U-1.13 (47.0)	L (55.0)	I-L (6.7)	I-L (44.5)	L (59.2)	I-0.05-U (14.7)	I-L (40.0)
p-hydroxy benzoate	L (50.7)	L (48.1)	U-0.62 (64.3)	L (44.0)	I-L (41.4)	L (51.0)	I-L (37.6)	I-L (35.5)
m-hydroxy benzoate	I-L (26.1)	I-L (39.9)	L (57.3)	U-0.33 (64.3)	L (64.3)	L (44.6)	L (51.0)	I-L (5.7)
Protocatechuate	U-2.06 (50.0)	U-1.13 (55.0)	I-0.62-U (34.6)	I-0.21-U (38.5)	I-0.42-U (21.3)	I-L (36.4)	I-0.3-U (29.2)	I-L (35.0)
Catechol	U-2.06 (54.0)	U-0.33 (44.0)	I-0.22-U (13.7)	I-0.37-U (6.4)	0	I-L (5.5)	I-0.18-U (10.2)	I-0.71-U (26.6)
Adipate	L (69.3)	L (47.4)	U-0.62 (61.8)	L (46.3)	U-1.0 (65.5)	L (58.5)	L (48.1)	I-L (36.0)
Succinate	I-0.22-U (35.0)	I-0.2-U (35.0)	I-0.22-U (32.0)	U-0.21 (73.3)	I-0.42-U (38.1)	I-0.53-U (32.0)	U-0.77 (44.6)	U-0.88 (52.3)
Acetate	U-0.15 (70.3)	U-0.25 (46.8)	U-0.27 (52.5)	U-0.1 (52.5)	U-0.42 (55.8)	U-0.53 (44.5)	U-0.3 (50.0)	U-0.64 (67.0)
2,4-dihydroxy benzaldehyde	I-0.38-U (11.1)	I-0.02-U (5.3)	I-0.07-U (14.7)	I-L (4.7)	I-0.15-U (19.1)	I-0.12-U (7.1)	I-0.05-U (9.8)	0
Salicylaldehyde	I-0.04-U (6.0)	0						
Citrate	L (61.0)							
α-ketoglutarate	I-L (36.0)							
Fumarate	U-1.3 (66.0)							
Malate	U-0.36 (50.0)							

cells were mixed with fine washed glass powder to make a thick paste, and grinded in a previously chilled mortor for five minutes. Approximately 5 ml more of 0.5 M potassium phosphate buffer, pH 7.0 was added and grinding continued for another 10 minutes. The cell debris were removed by centrifugation at 4^{o} at 16000 x g for 20 minutes.

(1) Oxidation of Benzaldehyde and related Compounds with the Cell-free Extract -

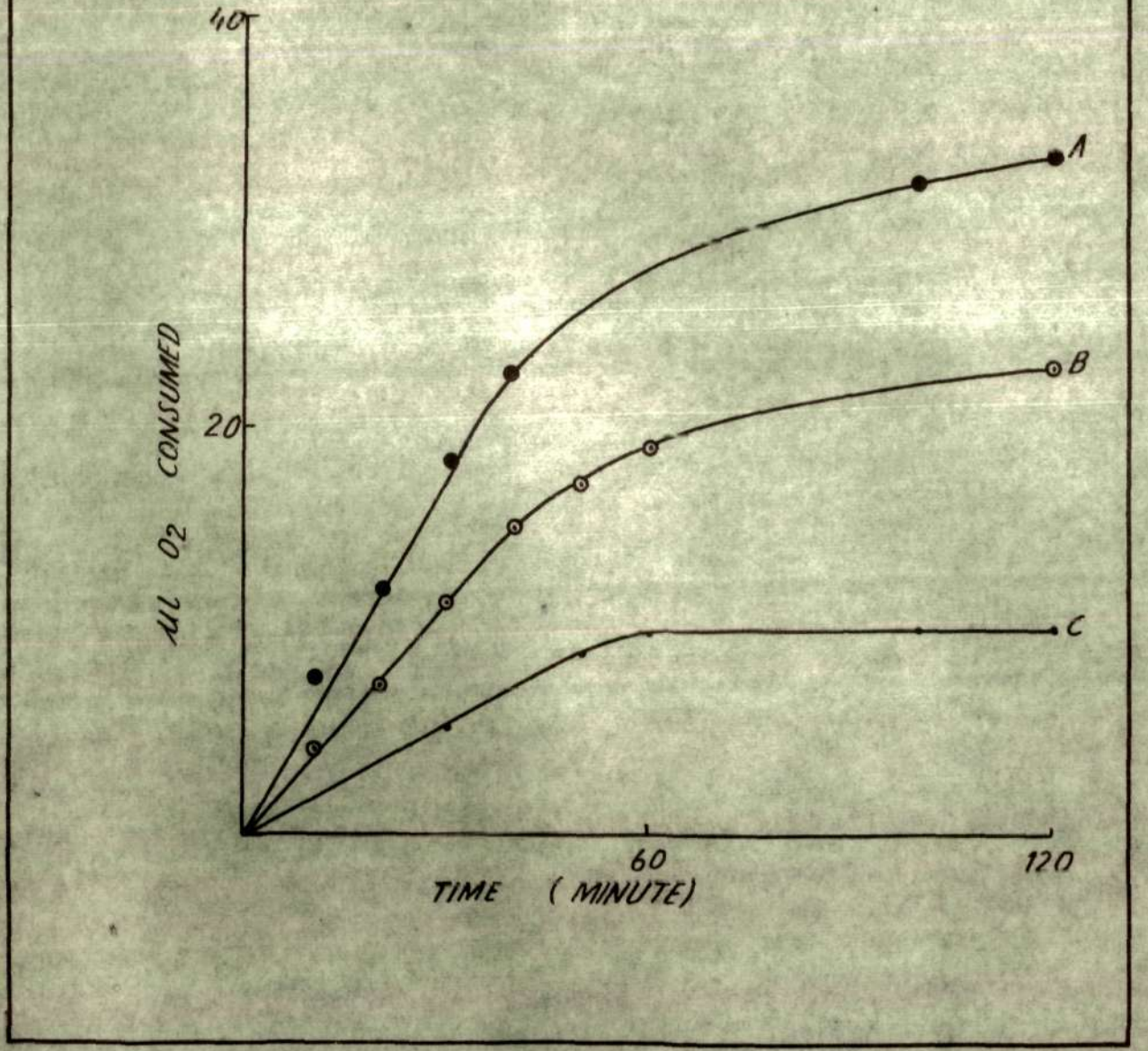
The ability of the cell-free extracts to oxidise benzaldehyde, benzoate, p-hydroxy benzoate, m-hydroxy benzoate, protocatechuate, catechol and 2,4-dihydroxy benzaldehyde was tested in the Warburg apparatus. It could only oxidise protocatechuate and catechol (Fig.45). This suggested that either the enzyme(s) involved in the oxidation of benzaldehyde, benzoate, p-hydroxy benzoate, m-hydroxy benzoate and 2,4-dihydroxy benzaldehyde has been inactivated during the preparation of cell-free extract or one or more of the enzymes responsible for the oxidation of these compounds are inducible. Both of these possibilities were ruled out by showing that mixing the cell debris with cell-free extract resulted in the oxidation of all

Fig.45. Oxidation of catechol and protocatechuate by cell-free extract of Achromobacter sp. Each Warburg flask in a total volume of 2.0 ml contained 355 µg equivalent of protein, 650 µ mole potassium phosphate buffer pH 7.0 and 2 µ mole substrate.

A ... Catechol

B ... Protocatechuate

C ... Endogenous


40
20
µl O2 CONSUMED
A
B
C
60
120
TIME (MINUTE)

these compounds (Fig.46). The cellular debris obtained from 100 mg cells were suspended in 12 ml of 0.5 M , potassium phosphate buffer, pH 7.0. Each Warburg flask received 1.1 ml of the suspended cell debris. This suggested that the factors necessary for the oxidation of these compounds are removed during the preparation of cell-free extract.

(ii) Evidence for the formation of β-Oxoadipate -

During oxidation of protocatechuate and catechol by cell-free extracts, β-oxoadipate was formed. This was shown by catalytic decarboxylation method of Sistrom and Stanier (1953). Using cell-free extract the total oxygen uptake for varying concentration of protocatechuate and catechol was estimated by conventional manometric technique. After completion of the reaction, varying aliquots of resulting incubation mixture were transferred to another Warburg flask and the amount of carbon dioxide liberated by addition of 4-aminoantipyrin was estimated by the direct method of Warburg. As shown in Table XI, for every mole of oxygen, one mole of β-oxoadipate was formed per mole of protocatechuate and catechol.

Fig.46. Oxidation of benzaldehyde and related compounds by cell-free extract of Achromobacter sp. in the presence of cellular debris. Each Warburg flask in a total volume of 2 ml contained 355 µg equivalent protein, cellular debris as described in text and a total of 800 µ mole potassium phosphate buffer pH 7.0 and 2 µ mole substrate.

A ... Protocatechuate

B ... Catechol

C ... m-hydroxy benzoate

D ... p-hydroxy benzoate

E ... Benzaldehyde

F ... Benzoate

G ... 2,4-dihydroxy benzaldehyde

H ... Endogenous

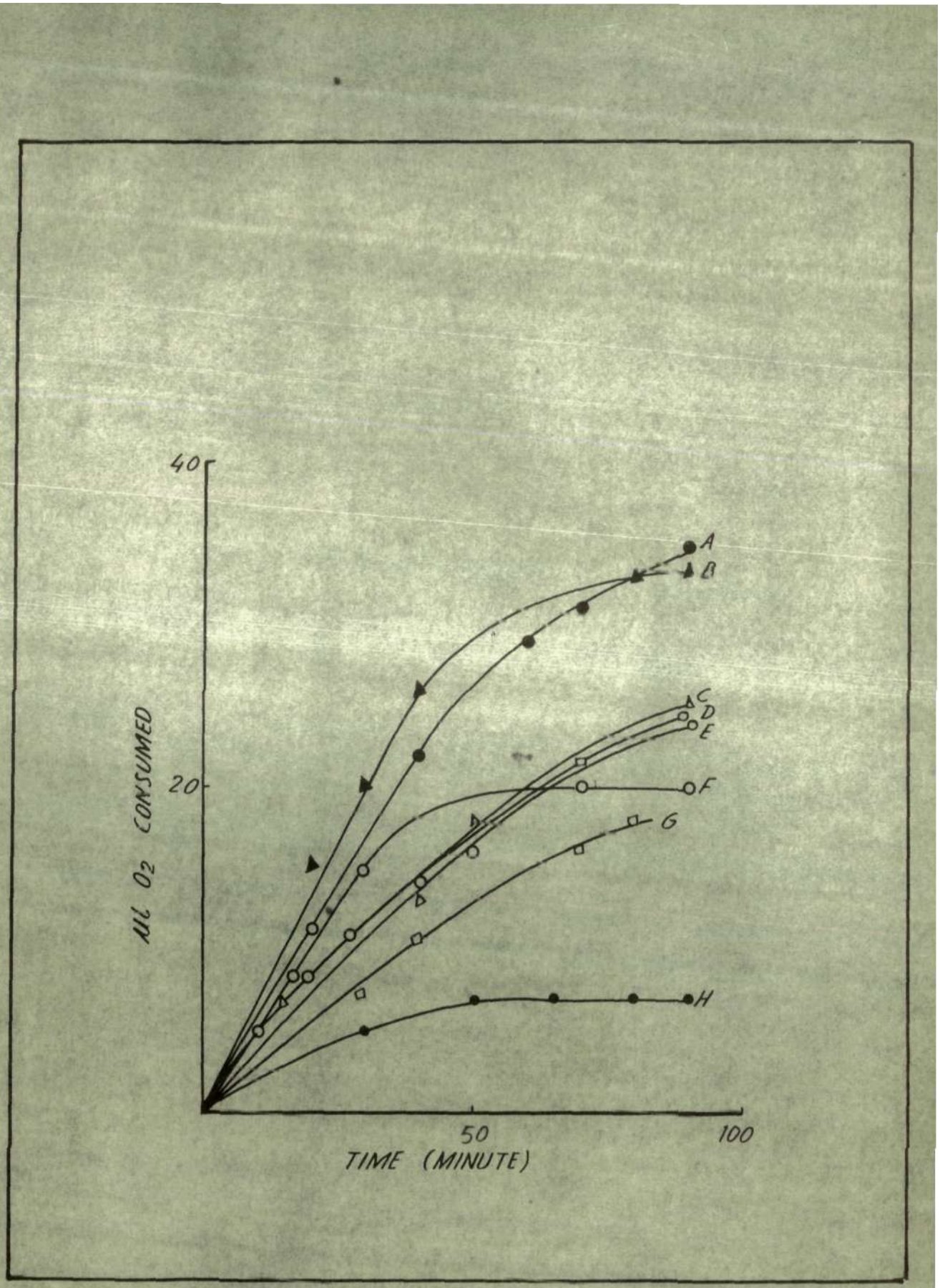

40
20
µl O2 CONSUMED
A
B
C
D
E
F
G
H
50
100
TIME (MINUTE)

TABLE - XI

Formation of β-oxoadipate during Oxidation of Protocatechuate and Catechol

Each Warburg flask contained 550 μ moles of potassium phosphate buffer pH 7.0 , substrate as shown in 0.2 ml in side arm, cell-free extract equivalent to 350 μg protein and water to a final volume of 1.6 ml. After four hours the total oxygen uptake and carbon dioxide evolved was determined from direct method of warburg. In separate warburg flasks suitable aliquots of the incubation mixture were transfered. To this was added 0.1 ml glacial acetic acid and suitable volume of 0.5 M potassium phosphate buffer, pH 7.0 to a final volume of 1.6 ml. From the side arm 0.4 ml of 0.1 M 4-aminoantipyrin was tipped and carbon dioxide liberated was estimated by direct method of warburg.

Substrate	Total Oxygen uptake	Total Carbon dioxide evolved before 4-aminoantipyrin addition	Total Carbon dioxide evolved after 4-aminoantipyrin addition
	(μ moles)	(μ moles)	(μ moles)
Protocatechuate (2 μ moles)	2.36	2.23	2.04
Catechol(2 μ moles)	2.18	0	2.04
Catechol(4 μ moles)	4.5	0	3.12

To confirm that the reaction product contains β-oxoadipate, Rothera test was performed on the resulting incubation mixture. The solution was saturated with ammonium sulphate followed by three drops of ammonia and few drops of 5% sodium nitroprusside. A pink colour was developed indicating the presence of the keto compound.

C. CHROMATOGRAPHY

(1) Accumulation of Intermediates - The oxygen uptake studies suggested that benzaldehyde is not completely oxidised and therefore it was suspected that intermediates involved in benzaldehyde oxidation may be accumulated in the culture broth.

Two litre of the supernatant liquid after 20 - 24 hours growth of the organism were concentrated under vacuum at 60 - 65° at pH 7.0 to 40 ml. The pH of the concentrated solution was adjusted to 2.0 with concentrated hydrochloric acid and extracted with peroxide-free ether in a liquid-liquid extractor for 72 hours. The ether was evaporated and the oily residue

was dissolved in 95% alcohol. Paper chromatography of a suitable aliquot in various solvents alongwith standard samples of phenolic compounds revealed the presence of p-hydroxy benzoic acid in the culture broth.

(ii) Studies with cell-free extract - In a total volume of 2 ml, 0.1 ml cell-free extract equivalent to 70 µg protein, 2 µ mole benzaldehyde, 1 µ mole $NADP^+$, 200 µ moles potassium phosphate buffer pH 7.0, were incubated in centrifuge tubes for varying times at room temperature (25-30°). The reaction was stopped at 0, 1, 2, 3 and 5 minutes by addition of 1 ml of 10% (w/v) trichloroacetic acid. The tubes were centrifuged and the supernatant thus obtained was adjusted to pH 2.0 with conc. HCl and extracted with peroxide-free ether in liquid-liquid extractor for 72 hours. The ether extract was dried overnight on anhydrous sodium sulphate, filtered and evaporated. The residue was dissolved in one ml of 95% ethanol. Thin layer chromatography and paper chromatography of this alcoholic solution along with the standard phenolic compounds revealed the presence of p-hydroxy benzoate, protocatechuate and catechol in the incubation mixture (Tables XII & XIII). Benzoic acid

TABLE - XII

Rf Values of Unknown Phenolic Compounds by Paper Chromatography.

Time Minutes	SOLVENTS ip: Am	Bu : py	COLOUR OF THE SPOT Detection Reagent Sulphanilic acid	POSSIBLE IDENTITY
0	-	-	-	-
1	0.22	0.31	Reddish brown	p-hydroxy benzoic acid
2	0.22	0.31	Reddish brown	p-hydroxy benzoic acid
	0.067	0.11	Light brown	Protocatechuic acid
3	0.24	0.32	Reddish brown	p-hydroxy benzoic acid
	0.06	0.12	Light brown	Protocatechuic acid
	0.80	0.93	Brown	Catechol
5	0.21	0.31	Reddish brown	p-hydroxy benzoic acid
	0.06	0.11	Light brown	Protocatechuic acid
	0.80	0.93	Brown	Catechol

TABLE - XIII

Rf Value of Unknown Phenolic Compounds by Thin Layer Chromatography

Time Minutes	SOLVENTS ip: Am	Bz : Ox	COLOUR OF THE SPOTS Detection Reagents Tetrazotized Benzidine	Sulphanilic acid	POSSIBLE IDENTITY
0	0.58	0.21	Yellowish brown	Yellow	Unidentified
1	0.37	0.60	Yellowish brown	Yellow	p-hydroxy benzoic acid
	0.58	0.21	Yellowish brown	Yellow	unidentified
2	0.58	0.21	Yellowish brown	Yellow	Unidentified
	0.38	0.60	Yellowish brown	Yellow	p-hydroxy benzoic acid
	0.037	0.52	Light Brown	Brown	Protocatechuic acid
3	0.58	0.60	Yellowish brown	Yellow	p-hydroxy benzoic acid
	0.037	0.54	Light brown	Brown	Protocatechuic acid
	0.59	0.68	Dark brown	Dark brown	Catechol
5	0.34	0.58	Yellowish brown	Yellow	p-hydroxy benzoic acid
	0.037	0.52	Light brown	Brown	Protocatechuic acid
	0.59	0.70	Dark brown	Dark brown	Catechol

was also identified in all the incubation mixtures by paper chromatography as described by Copius-Peereboom and Beekes (1964). In thin layer chromatography no attention was paid to the unidentified spot as it was also present at the zero time. The zero time samples were prepared by addition of trichloroacetic acid first followed by cell-free extract.

(iii) (a) Benzaldehyde dehydrogenase - The cells were grown in benzaldehyde broth and the cell-free extract was prepared in 0.5 M potassium phosphate buffer pH 7.0. The assay system used was essentially that of Friedman et al.,(1943). After deproteinization, the remaining benzaldehyde was estimated colorimetrically by the hydrazone formation. As shown in Table XIV, the enzyme is more specific for $NADP^+$.

Using the conditions of colorimetric assay it was established that there was co-linearity in the disappearance of benzaldehyde upto 30 minutes (Fig.47).

(b) Spectrophotometric method - The benzaldehyde dehydrogenase was also assayed spectrophotometrically at 340 mμ. Under the conditions of

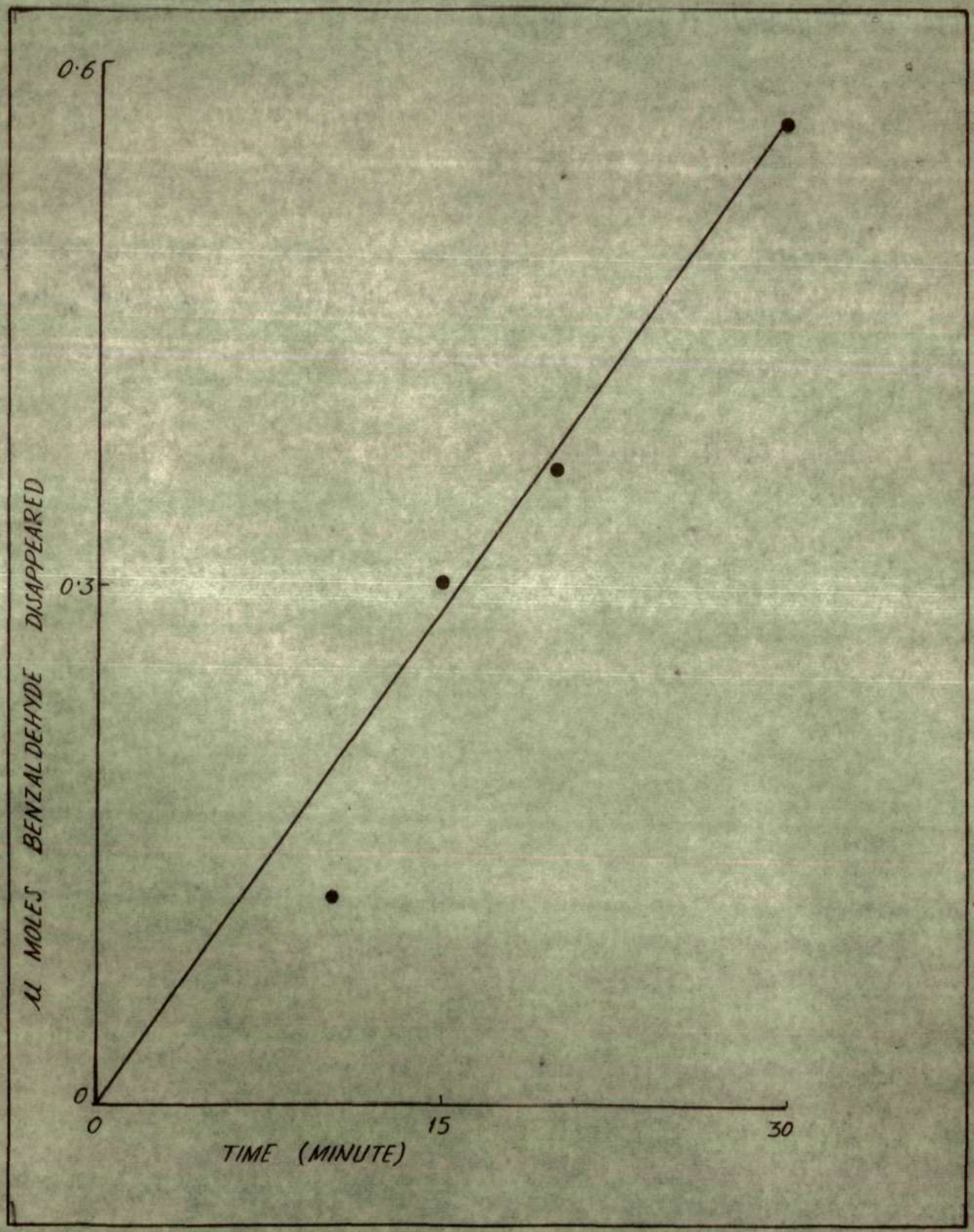
TABLE - XIV

Requirements for Conversion of Benzaldehyde to Benzoic acid

The complete system in a total volume of 1 ml contained, 1.18 µ moles benzaldehyde, 100 µ moles potassium phosphate buffer pH 7.0 and cell-free extract equivalent to 144 µg protein. Additions were made as indicated. After 30 minutes incubation at room temperature, 1 ml of 10% (w/v) trichloro-acetic acid was added to stop the reaction. After centrifugation, 1 ml supernatant was analyzed for benzaldehyde as described under experimental. The control was taken to correct for the endogenous oxidation. The values reported have been corrected accordingly.

SYSTEM	BENZALDEHYDE OXIDISED
	(µ Moles)
Complete	0.072
Complete + NAD^+ (1 µ mole) ...	0.290
Complete + $NADP^+$(1 µ mole) ...	0.600

Fig.47. Effect of time on benzaldehyde oxidation. The complete system in a total volume of 1 ml contained 1.8 µ mole benzaldehyde, 100 µ mole potassium phosphate buffer pH 7.0, 1 µ mole $NADP^+$ and cell-free extract equivalent to 144 µg protein.


0·6
0·3
0
μ MOLES BENZALDEHYDE DISAPPEARED
0
15
30
TIME (MINUTE)

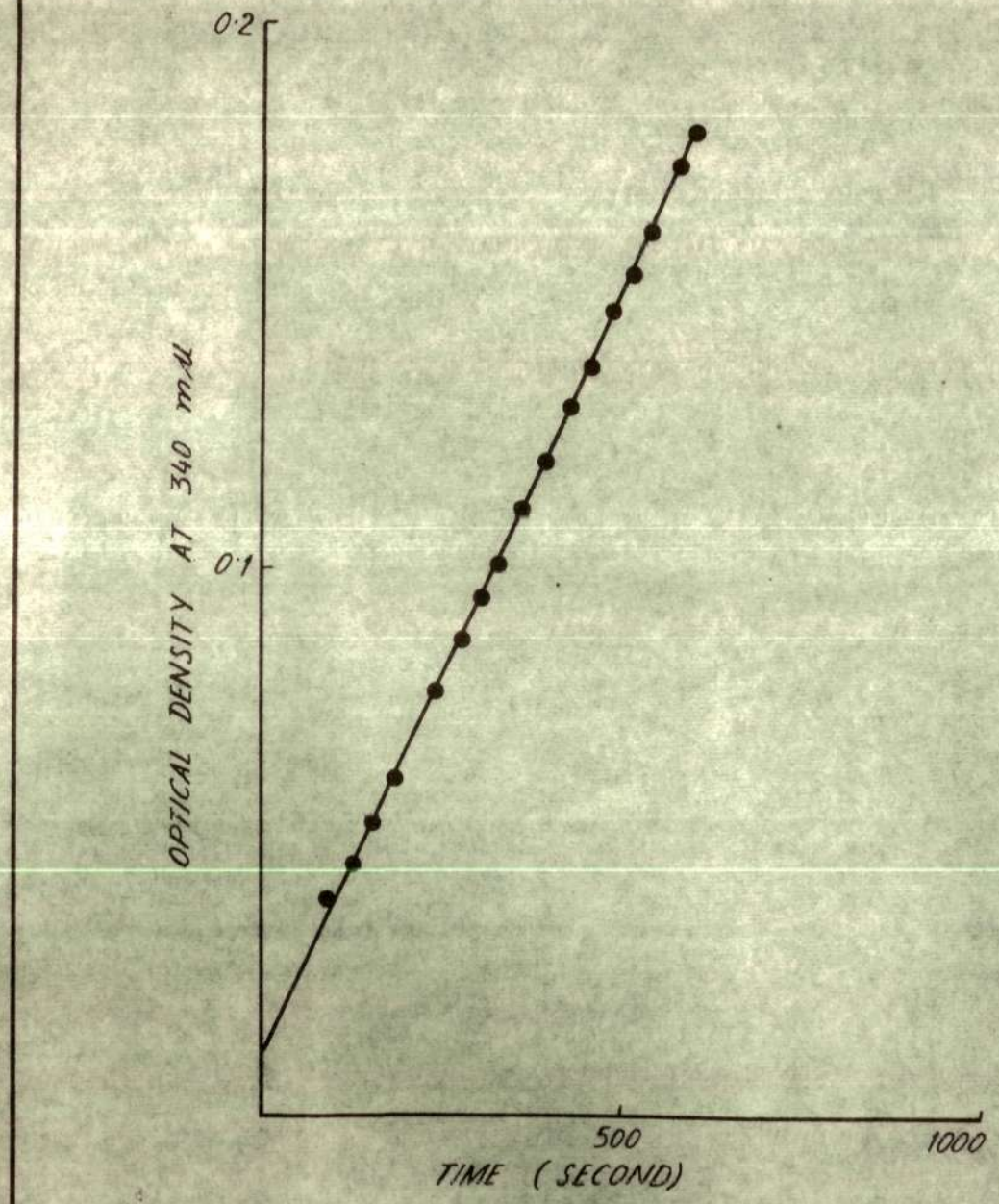
assay, one enzyme unit is defined as that amount of enzyme which brings about an O.D.change of 0.01 per hour (Fig. 48).

(c) Ammonium Sulphate Precipitation -The enzyme could be precipitated at 50% ammonium sulphate saturation. To 9.5 ml of cell-free extract (1762 enzyme units, specific activity 262) 2.97 gm ammonium sulphate was added in small amounts (about 100 mg) at an interval of 2 to 3 minutes. In order to prevent the denaturation of the enzyme, the beaker containing the enzyme was placed in another beaker having crushed ice. The solution was mechanically stirred with magnetic stirrer till all ammonium sulphate dissolved. After 30 minutes the contents of the beaker were centrifuged at 15000 r.p.m. The precipitate thus obtained was dissolved in one ml of 0.5 M potassium phosphate buffer pH 7.0. The enzyme (1 : 10) was assayed spectrophotometrically (1660 enzyme units, sp. activity 326).

(iv) Enzymic hydroxylation of p-hydroxy benzoate -

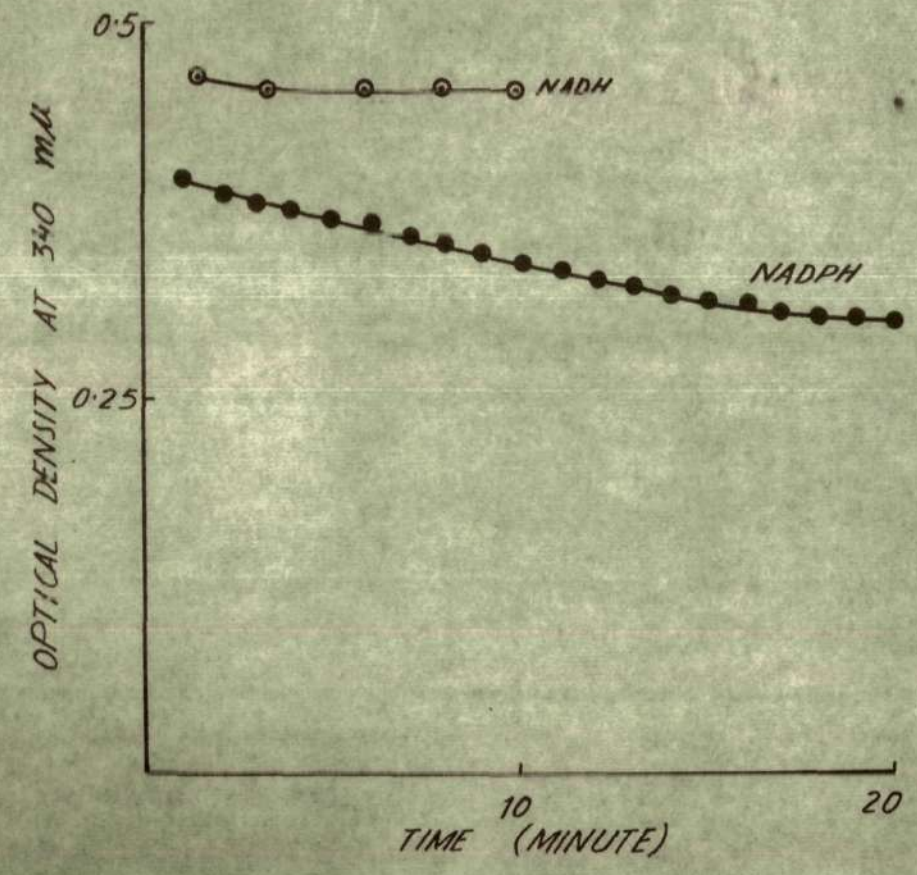
The crude cell-free extracts were also able to catalyse the NADPH dependent hydroxylation of p-hydroxy benzoate.

Fig.48. Spectrophotometric assay of benzaldehyde dehydrogenase. In a total volume of 2 ml, 1.85 µ mole benzaldehyde, 200 µ mole potassium phosphate buffer pH 7.0, 1 µ mole $NADP^+$ and cell-free extract equivalent to 71 µg protein were incubated at 30°. The reaction was initiated by addition of the cell-free extract.


0·2
0·1
OPTICAL DENSITY AT 340 mμ
500
1000
TIME (SECOND)

The conversion is strictly NADPH dependent (Fig.49). The disappearance of NADPH or NADH was spectrophotometrically followed at 340 mμ. In a total volume of 2 ml , 2 μ moles p-hydroxy benzoate , 200 μ moles , 0.5 M potassium phosphate buffer pH 7.0 , 2 μ moles NADH and cell-free extract equivalent to 140 μg protein were incubated at 30° and disappearance of NADPH was followed spectrophotometrically at 340 mμ. The reaction was initiated by addition of cell-free extract. Under the conditions of assay one enzyme unit is defined as that amount of enzyme which brings about an O.D. change of 0.01 per hour at 340 mμ. The cell-free extract contained 1950 enzyme units having specific activity of 274. In order to identify the reaction product of NADPH dependent hydroxylation of p-hydroxy benzoate, 4 μ moles p-hydroxy benzoate, 400 μ moles 0.5 M potassium phosphate buffer pH 7.0 ,2 μ moles NADPH and cell-free extract equivalent to 280 μg protein were incubated at 30° for 10 minutes in a total volume of 4 ml. The reaction was stopped by addition of 1 ml of 10% (w/v) trichloroacetic acid. The precipitated protein was removed by centrifugation and the supernatant extracted with peroxide free ether in liquid-liquid

Fig.49. Hydroxylation of p-hydroxy benzoate. The assay method is described in the text.


0·5
0·25
OPTICAL DENSITY AT 340 mμ
NADH
NADPH
10
20
TIME (MINUTE)

extractor for 48 hours. The ether extract was dried over night on anhydrous sodium sulphate, filtered , evaporated to dryness and the residue dissolved in 1 ml of 95% ethanol. The paper chromatography of this solution in ip : Am, Bu : py solvents showed the presence of protocatechuic acid and catechol. Under the same assay conditions if p-hydroxy benzoate is replaced by m-hydroxy benzoate, it utilizes NADPH but in contrast to p-hydroxy benzoate the activity is low (600 enzyme units; sp. activity 84). No attempt was made to identify the reaction product but it shows that hydroxylation phenomenon is not very specific.

VII. DISCUSSION

From the studies on the identification of the organism it was concluded that the organism belongs to the "Achromobacter sp."

From the Tables V, VI and VII it is evident that the optimal conditions for the cultivation of the organism are 30°, pH 7 and 0.05% (v/v) benzaldehyde. Ammonium sulphate and peptone are the best nitrogen sources (Table IV). The organism is aerobic since aeration favoured the growth of the organism (Table VII). On the basis of the Molar Growth Yield, the compounds used to support the growth of the organism may be divided into three groups (Table III). Among the first group may be kept benzaldehyde, benzoate, p-hydroxy benzoate and adipate with which the Molar Growth Yield was maximum. The second group (acetate, citrate, succinate and 2,4-dihydroxy benzaldehyde) had equally lower Molar Growth Yield. The third group (2,4-dihydroxy benzoate, m-hydroxy benzoate, salicylaldehyde and salicylate) had the lowest yield. Each group of the compounds yielded equal biologically useful energy to the organism. Appreciable time lags observed during the growth of the organism on p-hydroxy benzoate

(60 minutes), m-hydroxy benzoate (60 minutes), 2,4-dihydroxy benzaldehyde (30 minutes), glucose (60 minutes), citrate (30 minutes), acetate (30 minutes), succinate (60 minutes), adipate (60 minutes), presumably indicate that permeability and induction of enzyme(s) may be the important factors for the quantitative difference in growth rates. The quantitative differences in growth rates observed with the various substrates (Table IX) were frequently reflected in the rates of oxygen uptake with washed cell suspensions (Table X). For the experiments described in Table X, the organism were grown on as many of the likely intermediates which supported growth, and then exposed in the Warburg apparatus to similar compounds. The resulting patterns of oxygen uptake indicated the degree to which non-specificity of enzyme induction and activity contributed to the economy of the cell. The pattern of oxygen uptake obtained with benzaldehyde-grown cells indicated that benzoate, protocatechuate, catechol, succinate and acetate (Figs. 5,6) may be on the direct pathway of benzaldehyde oxidation. The oxidation of citrate, α-ketoglutarate, malate and fumarate (Fig.8) by the benzaldehyde-grown cells is

a fair indication of the fact that the final oxidation of benzaldehyde is achieved through Tricarboxylic Acid Cycle. The lag in the oxidation of p-hydroxy benzoate, citrate and ∝-ketoglutarate may be due to the problem of induction and permeability. Although 2,4-dihydroxy benzaldehyde and salicylaldehyde were oxidized (Fig.6), the percent oxidation of these compounds was very poor. This indicated the non-specificity of the enzyme(s) involved in the oxidation of hydroxy substituted benzaldehydes. Similarly the percent oxidation of benzaldehyde and 2,4-dihydroxy benzaldehyde was very poor when benzoate-grown cells were used. These cells did not oxidize salicylaldehyde. Therefore these compounds may not be on the direct route of benzoate utilization. Since protocatechuate, catechol, succinate and acetate(Figs.18,19) did not show any lag, these compounds could be on the direct route of benzoate catabolism. In accordance to "Sequential induction" theory, p-hydroxy benzoate-grown cells oxidized benzaldehyde, benzoate and m-hydroxy benzoate with lag(Fig.21). Since protocatechuate, catechol, succinate and acetate were oxidized without lag by p-hydroxy benzoate-grown cells(Figs.22,23)and m-hydroxy benzoate-grown cells (Figs. 25,26), these compounds may be considered on the

direct pathway for the utilization of p-hydroxy benzoate and m-hydroxy benzoate. Except 2,4-dihydroxy benzaldehyde, the acetate-grown cells oxidised all the aromatic compounds tested with lag (Figs. 30,31,32). However , in comparison to benzaldehyde-grown cells, the initial rate as well as percent oxidation of 2,4-dihydroxy benzaldehyde was poor. When succinate-grown cells were used, all the aromatic substrates were oxidized without lag but the initial rate as well as percent oxidation was poor (Figs.33,35). There was lag in the oxidation of p-hydroxy benzoate and m-hydroxy benzoate (Fig.34). The glucose-grown cells oxidized benzoate, p-hydroxy benzoate, m-hydroxy benzoate and protocatechuate with lag (Figs. 37,38) but benzaldehyde, catechol, succinate and acetate were oxidized without lag (Figs. 36, 38). Similarly the adipate-grown cells oxidized benzaldehyde , 2,4-dihydroxy benzaldehyde, protocatechuate, succinate and acetate without lag (Figs. 27, 29). These results suggested the non-specificity and inducibility of the enzyme(s) involved in bacterial oxidation of benzaldehyde.

In order to study the patterns of enzyme induction, protein synthesis inhibitors were used. It is very difficult to avoid the enzyme induction even in the

non-proliferating suspensions, if the turn over of the enzyme is slow and the cells are previously adapted to the compound. The puromycin and chloramphenicol inhibit the protein synthesis at the translation level but it is not known at what stage protein synthesis is inhibited by the o-nitrobenzoic acid (Montgomery and Durham,1970). Puromycin was an effective protein synthesis inhibitor for the benzaldehyde-grown cells. It inhibited the rate as well as total oxygen uptake for all the benzaldehyde-grown cells. In the benzaldehyde-grown cells, the enzymes necessary for the catabolism of benzaldehyde and its intermediates are inducible. The lag in the oxidation of p-hydroxy benzoate by benzaldehyde-grown cells was prolonged from 20 to 40 minutes in presence of puromycin (0.3 mM). This increase in the lag in the oxidation of p-hydroxy benzoate may probably be due to the effect of inhibitor in the permease system responsible for the influx of the substrate molecule. In presence of 0.3 mM puromycin the lag in the oxidation of p-hydroxy benzoate and m-hydroxy benzoate was prolonged but 0.5 mM chloramphenicol and 10 mM o-nitrobenzoic acid had no effect. Since the cell-free extract of benzaldehyde-grown cells could oxidize both hydroxy benzoates, it was concluded

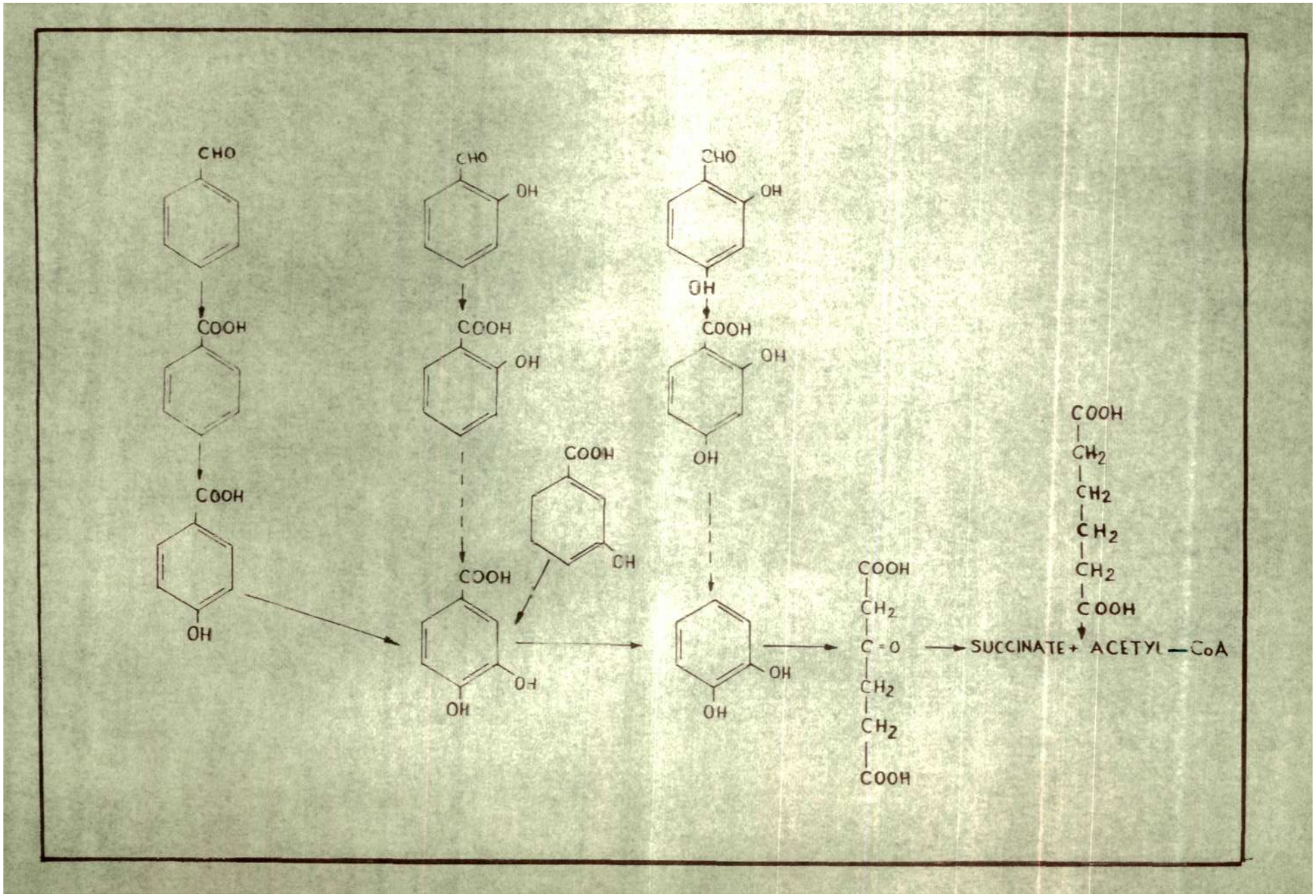
that permeability may be a limiting factor in the oxidation of these compounds by benzaldehyde-grown cells. In case of glucose-grown cells the total oxygen uptake of benzaldehyde and p-hydroxy benzoate increased in presence of puromycin (0.3 mM) and o-nitrobenzoate (10 mM). The increase in the total oxygen uptake may be either due to the oxidation of inhibitors or some impurity present in the inhibitor. In the presence of puromycin and o-nitrobenzoate the lag phase in the oxidation of p-hydroxy benzoate was prolonged from 25 minutes to 85 and 120 minutes respectively. According to "Sequential induction" theory of Stanier, the synthesis of enzymes responsible for utilisation of intermediate products of benzaldehyde should be immediately induced in the presence of the growth substrates. In presence of chloramphenicol, the initial rate as well as total oxygen uptake of benzaldehyde decreased but that of p-hydroxy benzoate and m-hydroxy benzoate was completely supressed. Therefore, unlike puromycin and o-nitrobenzoate, chloramphenicol acted as inhibitor for the synthesis of induced enzyme(s). The oxidation of benzaldehyde by glucose-grown cells without-lag may be an indication of the constitutive nature of initial enzyme(s) involved in the oxidation of

benzaldehyde because benzaldehyde-grown cells not only oxidized benzaldehyde at a rate but the total oxygen uptake was higher than glucose-grown cells (Figs.6,38). Using cell-free extracts of benzaldehyde-grown cells evidence was obtained for the formation of p-hydroxy benzoate protocatechuate and catechol from benzaldehyde. Within 1 minute of incubation of benzaldehyde in presence of $NADP^+$, p-hydroxy benzoate was formed. At 2nd minute protocatechuate appeared followed by catechol at 3rd minute (Table XII and XIII). In the presence of 4-amino-antipyrin β-oxoadipate is well known to decarboxylate to liberate equimolar quantities of carbon dioxide (Sistrom and Stanier, 1953). The cell-free extracts of benzaldehyde-grown cells catalysed oxidation of protocatechuate and catechol. As shown in Table XI, 1.18 and 1.09 mole of oxygen was respectively consumed with one mole of protocatechuate and catechol. With former 1.1 mole of carbon dioxide was also liberated. The resulting incubation mixture gave 1.02 mole of carbon dioxide in presence of 4-aminoantipyrin in each case. Thus in total protocatechuate consumed 1.18 mole oxygen and liberated 2.12 moles of carbon dioxide whereas catechol used 1.09 mole of oxygen and liberated 1.02 mole of carbon

dioxide in presence of 4-aminoantipyrin. Furthermore in both cases the final incubation mixture gave positive Rothera test (Hawk et al.,1948). These studies confirmed the formation of β-oxoadipate by cell-free extracts of benzaldehyde-grown cells with either protocatechuate or catechol as substrates.

The pathway for the catabolism of benzaldehyde and related compounds by the Achromobacter sp. is shown in the Fig.50. The particular catabolic route taken by a microorganism capable of utilizing an aromatic compound, presumably, depends on its genetic potential, coupled with the influence of environment for the synthesis of relevent enzymes. Evans (1947) has reported oxidation of benzoate to protocatechuate through p-hydroxy benzoate or m-hydroxy benzoate by Vibrio 01. He suggested further oxidation of protocatechuate before the ring cleavage but he did not identify this intermediate. Proctor and Scher (1960) in the study of metabolism of benzoate by a Rhodopseudomonas strain have reported the formation of protocatechuate, catechol and ∝ -oxoacid. The benzoic acid was directly metabolized to these intermediates without the involvement of monohydroxy derivatives of benzoic acid. The benzoate

Fig.50. The metabolism of Benzaldehyde and related compounds by Achromobacter sp.


CHO
COOH
OH
CH
CH2
C=O
SUCCINATE + ACETYL—CoA

metabolism is also reported by Bernheim (1942), Sheperd and Villanueva (1959) and Bhat et al., (1959). The Achromobacter sp. is metabolizing the benzaldehyde to catechol through benzoate, p-hydroxy benzoate and protocatechuate. This is also evident from the oxygen uptake studies (Table X) and the chromatographic studies (Table XII, XIII). All these intermediates have been identified in the oxidation of benzaldehyde by the cell-free extract. The lag phase with p-hydroxy benzoate by the whole cells may be due to induction of the permease. The cell-free extract oxidize NADPH in presence of p-hydroxy benzoate (Fig.49). The paper chromatography of the resulting incubation mixture showed the presence of protocatechuate , which confirmed the hydroxylation of p-hydroxy benzoate. In the mandelate metabolism by P.fluorescens, Stanier et al.,(1950) have shown that benzaldehyde which was an intermediate in mandelate metabolism was degraded through benzoate and catechol. Sleeper et al.,(1950) reported that benzoate-grown P.fluorescens, oxidized catechol without lag but there was lag in the oxidation of protocatechuate. Similar types of results are also reported by Kennedy and Fewson (1968). The pathway followed by the Achromobacter sp. appears to be

different from the pathways reported by the earlier workers but it has certain similarities with the pathways recognized in Arthrobacter sp. (Mulla Khan Bhai and Bhat, 1966). One of the pathway followed by this organism was through the intermediates of p-hydroxy benzoate, protocatechuate, catechol and pyruvate. The ring cleavage product of Achromobacter sp. from catechol was β-oxoadipate which in turn cleaved to acetate and succinate.

A $NADP^+$ dependent and another NAD^+ dependent benzaldehyde dehydrogenase have been reported (Gunsalus et al., 1953; Jamaluddin et al., 1970). The benzaldehyde dehydrogenase present in the Achromobacter sp. is $NADP^+$ dependent (Table XIV). The mixed-function oxidase catalysing the hydroxylation of p-hydroxy benzoate is NADPH specific (Fig.49). However, it is not yet clear whether the hydroxylating enzyme of Achromobacter sp. contains FAD as shown for Pseudomonas sp(Hosokawa and Stanier, 1966).

The mechanisms for the regulation of enzymes induced by aromatic substrates in bacteria have recently received considerable attention (Mandlestam and Jacoby , 1965; Stevenson and Mandlestam, 1965; Hegeman, 1966; Ornstan, 1966) and attention has been drawn in micro-

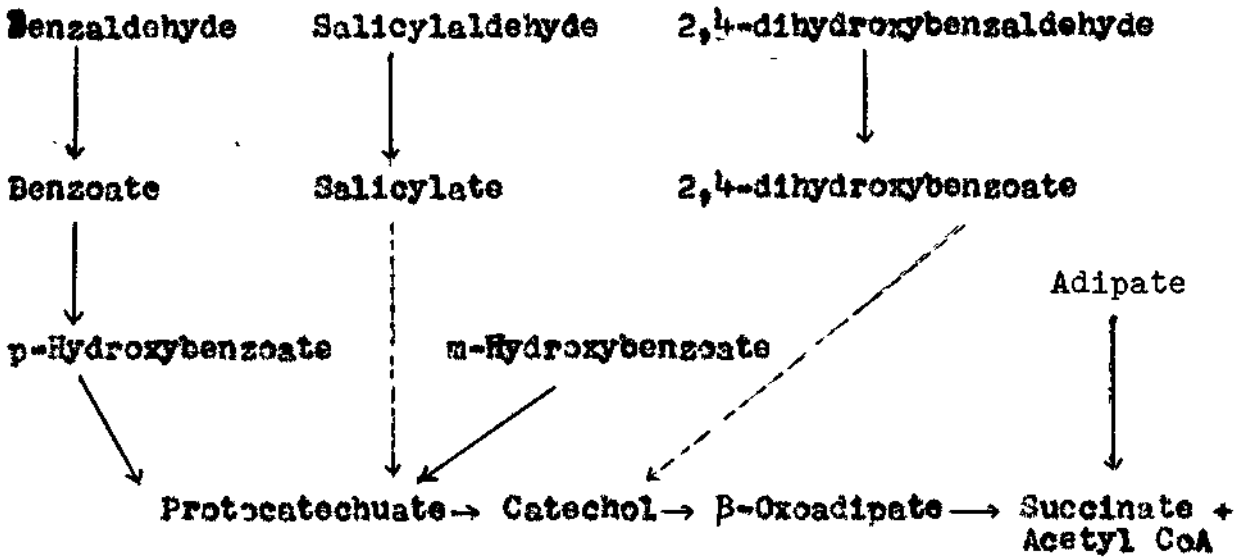
organisms the presence of multienzyme sequences induced by, and active on a range of related substrates. The glucose-grown cells also oxidized benzaldehyde immediately. This type of non-specificity has advantage, especially in soil where a particular environment may contain a number of different analogues of benzaldehyde, all formed by the degradation of lignin (Stevenson, 1964). The possible ecological significance of partial degradation such as salicylaldehyde to salicylic acid and 2,4-dihydroxy benzaldehyde to 2,4-dihydroxy benzoic acid should not be overlooked. Not only might this type of transformation be capable of providing energy, even if not serving as a carbon supply, but the product might be acted upon by other organisms.

VIII. SUMMARY

An organism capable of utilizing benzaldehyde as sole source of carbon has been isolated from the local soil by enrichment culture technique. The organism belongs to the genus "Achromobacter"

In order to understand the mechanism of benzaldehyde degradation, the oxidation of benzaldehyde and a number of related compounds were investigated by the technique of sequential induction. These compounds included benzaldehyde, 2,4-dihydroxy benzaldehyde, salicylaldehyde, benzoate, p-hydroxy benzoate, m-hydroxy benzoate, protocatechuate, catechol, adipate, citrate, α-ketoglutarate, succinate, fumarate, malate, acetate and glucose. The organism was grown on each available compound, harvested and the oxygen uptake studied manometrically. These studies suggested that some of the enzymes are constitutive while others are inducible. Benzoate, p-hydroxy benzoate, protocatechuate, catechol and β-oxoadipate have been identified on in vitro incubation of the cell-free extract with benzaldehyde, protocatechuate or catechol.

The dissimilation of benzaldehyde by the Achromobacter sp. has been shown to follow the following converging pathway.



Since the benzaldehyde-grown cells metabolized the intermediates of the tricarboxylic acid cycle, further utilization of β-oxoadipate may occur through the Kreb Cycle. The cell-free extract were shown to (i) posses $NADP^+$ dependent benzaldehyde dehydrogenase and (ii) catalyse NADPH dependent hydroxylation of p-hydroxy benzoate.

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